PREPARATION OF 7-(P-SUBSTITUTED-BENZYLAMINO)-4-NITROBENZOXADIAZOLE DERIVATIVES AND THE STUDY OF THEIR FLUORESCENT PROPERTIES IN SOLUTION

An abstract of a Thesis by
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The problem. The objective of this study was to synthesize a series of derivatives of 7-(benzylamino)-4-nitrobenzofurazan and study their fluorescent behavior in aqueous-nonaqueous solvent systems. The parent compound has been used by others as a fluorescent probe to determine the conformation changes of bovine trypsinogen and bacterial libosomes. Fluorescent probes have been used to: (1) establish the degree of polarity of a particular region of a protein; (2) measure distances between groups in a protein; (3) determine the extent of a flexibility of a protein; and (4) measure the rate of very rapid conformational transitions. By selecting those fluorescent probe molecules that have structural features similar to an enzymic substrate molecule, it is expected that the probe molecule will exhibit an affinity for the enzyme. Thus, it may be expected that the probe molecule will be specifically inserted into enzymes to reveal facets of their structure and dynamics.

Findings. This study resulted in the characterization of two new compounds, 7-(p-methylbenzylamino)-4-nitrobenzofurazan (BBD-CH_3) and 7-(p-chlorobenzylamino)-4-nitrobenzofurazan (BBD-Cl) which may have use as fluorescent probes. The quantum yield studies of this study indicated that the para substituent makes these compounds more sensitive to solvent polarity. No NMR spectra have been reported in the literature. The NMR spectra obtained in this study gave additional identification of these compounds. The changes in quantum yields and wavelengths of maximum emission with solvent polarity changes are reminiscent of other fluorescent probes which are important in protein conformation studies.

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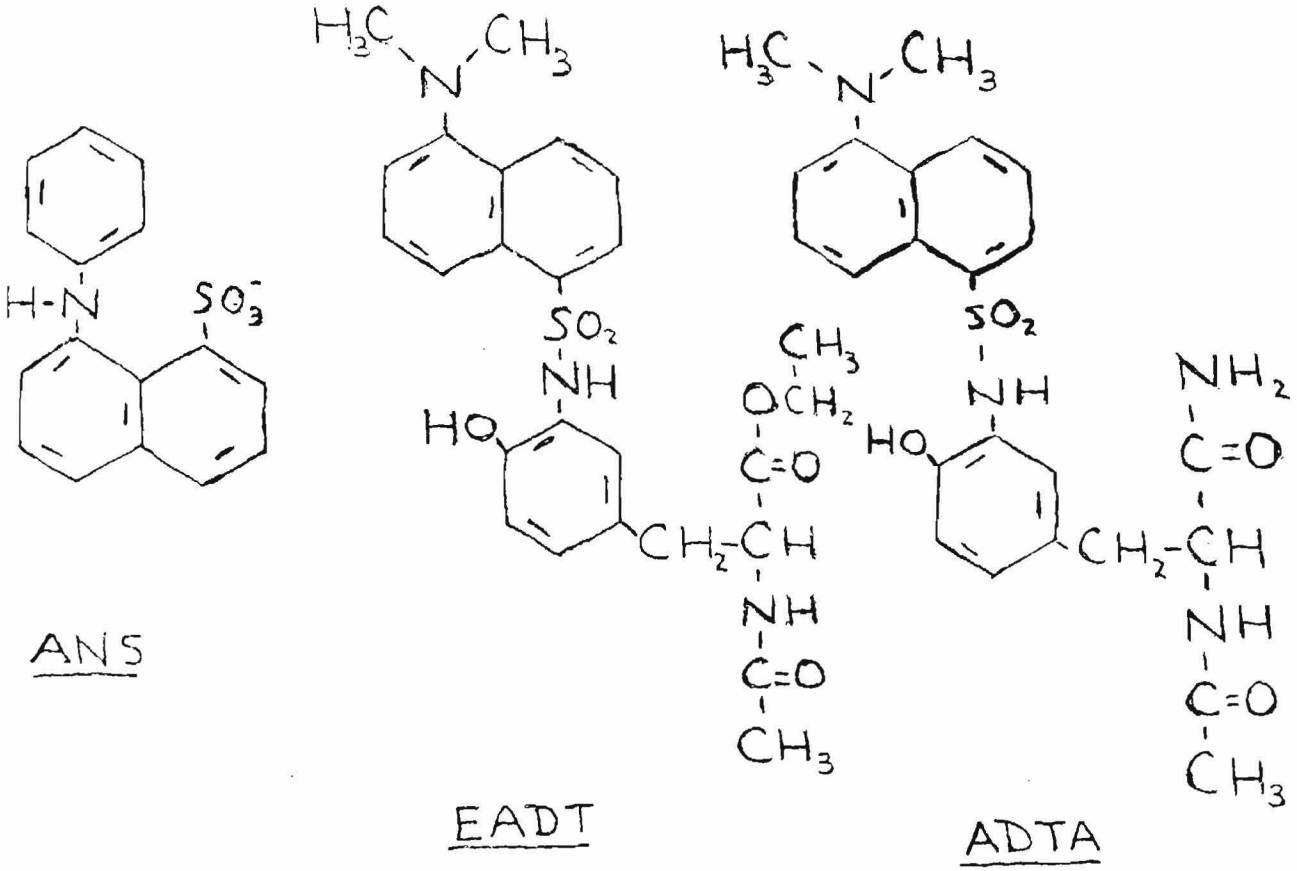
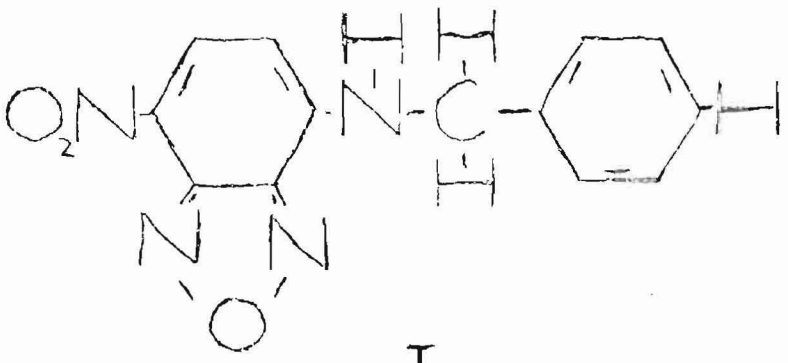
INTRODUCTION

Fluorescent probes have been defined as "small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with a protein or other macromolecule." (1) Although there are a significant number of fluorescence probes used at this time. They have similar structures to each other which will not allow them to bend to the sterically controlled binding sites on certain macromolecules. An example of this is 1-anilinonaphthalene-8-sulfonate, ANS, which can be used in studies of serum albumin, alcohol dehydrogense, or aponyoglobin that have highly or moderately nonpolar binding sites. This compound cannot be used in studies of chymotrypsinogen which has a highly polar active site. (2)

To be used as a fluorescent probe in proteins, the compound must be highly sensitive to polarity. An example is EADT [ethyl α-N-acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinate], and the corresponding amide, ADTA [α-N-acetyl-3-(1'-dimethylaminonaphthalene-5'-sulfonamido)tyrosinamide] which are used as model compounds of fluorescent "dansyltyrosyl" reporter groups in proteins. Their fluorescence shows a high quantum efficiency which is extremely sensitive to solvent polarity. (3)

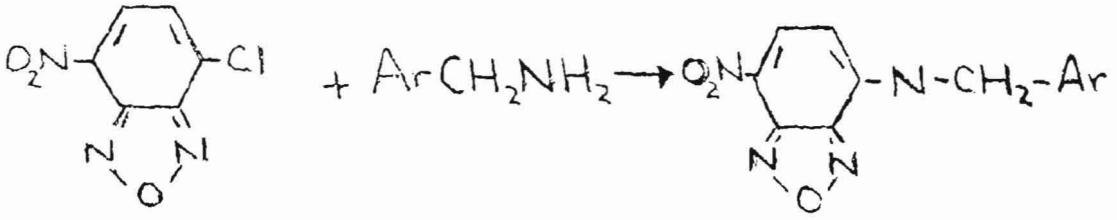


A new type of fluorescent probe has been prepared and characterized by Kenner and Aboderin. (4) Given the chemical nature of these compounds, analogs of the parent compound, 7-(benzylamino)-4-nitrobenzoforazan,(7-(benzylamino)-4-nitrobenzoxadiazole), I, can be synthesized.



I

The analogs may be synthesized by reacting 7-chloro-4-nitrobenzofurazan and p-substituted benzylamines.



II

The purpose of this study was to synthesize a series of derivatives of 7-(benzylamino)-4-nitrobenzofurazan and study their fluorescent behavior in aqueous-nonaqueous solvent systems. Studying these compounds in solvent systems of different polarity, provides information as to whether these compounds could be used as probes for hydrophobic regions in proteins.

Fluorescence Spectroscopy

A molecule excited to an upper electronic state by absorption of light can return to the ground state in a number of ways. Some of the possible fates of a molecule in the excited state are (i) fluorescence, a transition to the ground state accompanied by emission of a photon, (ii) internal conversion, a return to the ground state without radiation, (iii) intersystem crossing, a transition to an excited triplet state in which the electron spins are no longer paired. A molecule in the triplet state may return to the ground state in a radiation process termed

phosphorescence or it may return without emitting radiation. Alternatively, a molecule in the lower excited state and/or the triplet state may transfer excitation energy to chromophores or participate in photochemical reactions. (6) Figure 1 depicts these processes.

The interaction of a molecule in the excited state with one which absorbs the excitation energy efficiently upon collision, but does not fluoresce is not completely understood. The molecule that absorbs the energy is called a quencher. The energy of the quencher ultimately goes to the solvent, most probably through a series of very fast vibrational changes. The excited molecule can also be deactivated by collision with solvent molecules if no quencher molecules are present.

The time scale of these processes has important experimental consequences. In the absence of nonradiative processes, the lower excited state typically has a lifetime of a few nanoseconds whereas the triplet state usually has a lifetime between a millisecond and several seconds. The long lifetime of the triplet state makes it highly vulnerable to quenching processes, particularly those that are limited by diffusion. The result is that phosphorescence is seldom observed except in rigid media. In contrast, chromophores in fluid solution at room temperature are often fluorescent. The fact that fluorescence measurements can be made under a wider range of conditions (concentration,

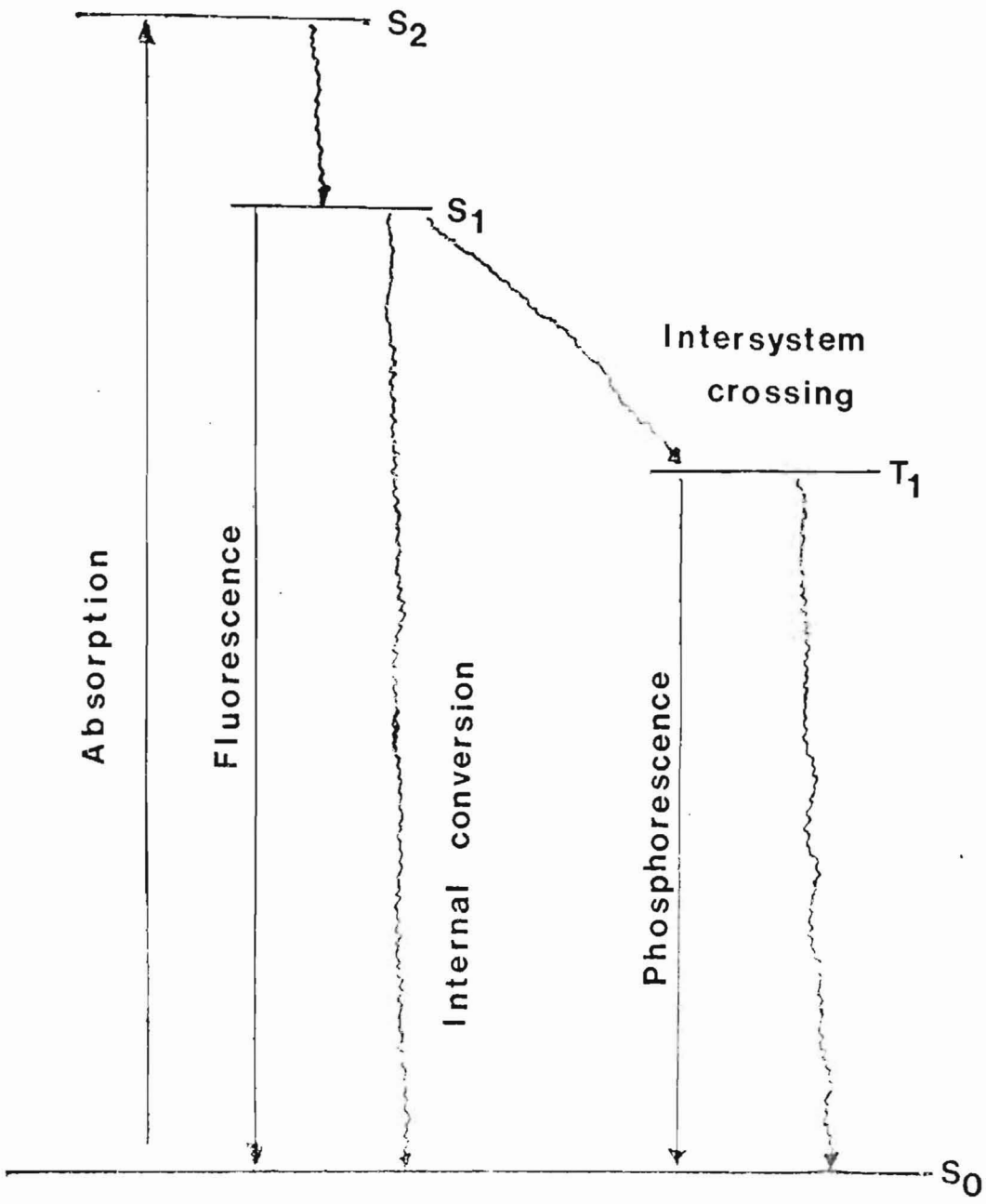


Figure 1. Excited-state processes. Straight arrows denote processes in which a photon is emitted or absorbed; wavy arrows denote transitions which do not emit radiation.

temperature, and solvent) than phosphorescence measurements accounts for the more extensive use of fluorescence spectroscopy. (2) Figure 2 is a diagram of a spectrofluorometer.

Two types of fluorescence spectra may be determined, an excitation spectrum or an emission spectrum. The fluorescence excitation spectrum (the relative efficiency of different wavelengths of exciting radiation to cause fluorescence) of a substance is obtained by measuring the intensity of the fluorescence as a function of the wavelength of excitation. Since fluorescence is directly proportional to the intensity of exciting light times quantum yield, then scanning a dye solution at low concentration with the excitation monochromator while holding the fluorescent monochromator at the wavelength of maximum fluorescence will give a spectrum that is directly proportional to ε, the molar absorptivity. This assumes quantum yield is independent of wavelength, which is not always the case. This method is a very sensitive means of measuring the absorption spectrum of a fluorescent substance.

Whereas absorption measurements are good only to a concentration of about 10^{-5}M, fluorescence excitation spectra may be determined for solutions with concentrations as low as 10^{-8}M. A major advantage of this technique is that it may be used for the spectrophotometric analysis of solutions containing two substances, both having similar absorption spectrum. Provided only one is fluorescent, the excitation

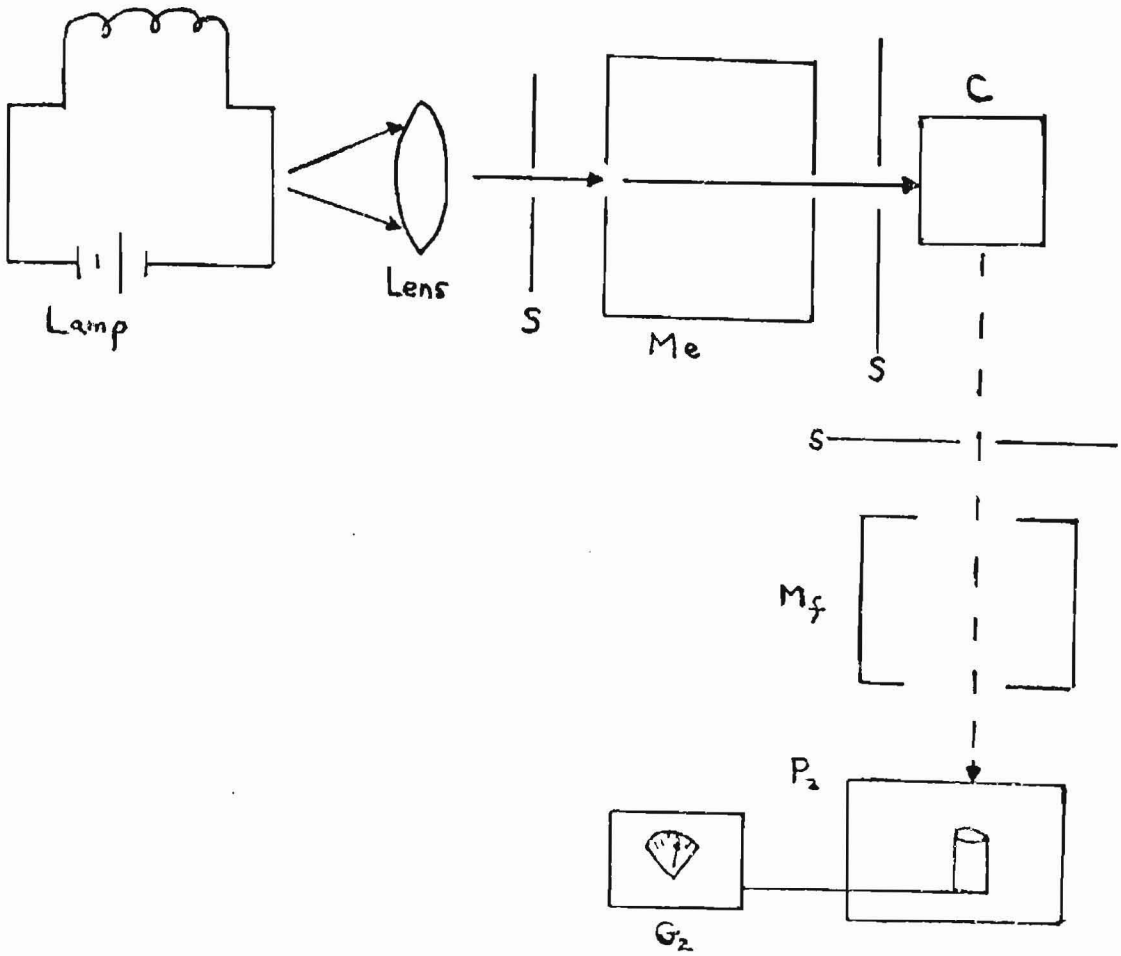


Figure 2. Spectrofluorophotometer. S = variable slit; M_e = excitative monochromator; c = sample cell; M_f = fluorescent monochromator for scanning the fluorescence spectra; if one wishes only to measure intensities, a filter excluding exciting light but passing fluorescent light may be used: P_2 = photo cell; G_2 = galvanometer to show output from P_2. (Reference 5)

spectrum will then be proportional to the concentration of the fluorescent substance.

A fluorescence emission spectrum is obtained by exciting a solution with light of a constant frequency and intensity, while scanning the quantum output of fluorescence. The fluorescence emission spectrum that is observed is not only a function of emitted light but also of the transmittance of the fluorescence monochromator which is a function of the wavelength of emitted light.

The excitation and emission spectra recorded are only approximate curves and do not represent the true spectra. The instrument can be corrected by determining the correction factors for the spectrofluormeter by comparing the emission curve on the instrument with that obtained with certain fluorescent dyes on a standardized instrument. The spectra in this study are uncorrected.

The quantum yield of fluorescence is defined as the ratio of quanta emitted to quanta absorbed:

$$\text{Quantum yield} = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} .$$

It is independent of the energy or wavelength of the quanta and thus differs from the energy efficiency of fluorescence.

The quantum yield of a fluorescent dye is determined either by a direct absolute measurement or by comparison to a reference compound. The direct method is usually the more desirable but is subject to greater experimental error. The

comparative approach is normally dependent upon other work in which a direct measurement has been made. It is most common to use a pure compound whose quantum yield and spectrum are well established. This method is by far the simplest for obtaining quantum yields. It consists of simply comparing the total fluorescence, i.e., the area of the emission peak, of the compound of interest with the total fluorescence of the reference compound. Comparison of the fluorescence intensities may also be used if it can be demonstrated that the intensity ratio of observed fluorescence is the same as the ratio of the areas of the fluorescence peaks.

Fluorescence spectroscopy is one of the most versatile and sensitive methods for physical studies of protein systems. The study of the binding properties of a number of proteins and nucleic acid polymers has grown at a rapid rate with the use of fluorescence spectroscopy. Considerable information concerning the nature of protein binding mechanisms and the intrinsic structure of proteins has been the result. It has been used to follow protein denaturation, conformation transitions, changes in subunit association, to characterize binding processes, to measure the polarity of binding sites, and orientation of ligands. Such studies are discussed in review articles by Velick, Steiner and Edelhock, Chen, Parker, Edelman and McClure, Stryer, and Brand and Gohlke. (6) Stryer (2) was the first

to note the specificity of ANS for hydrophobic regions of functional interest on macromolecular surfaces. The correlation between the spectroscopic properties of ANS in solvent system of varying polarity suggested that ANS and its analogs might be useful probes for regions of low polarity on the surface of proteins. Several halogenated derivatives of fluorescein have been used to characterize the active site of horse liver alcohol dehydrogenase. (7) Weber and Lawrence (8) were the first to observe conformational changes in proteins using fluorescent probes (N-phenyl-substituted naphthalene dyes in the presence of heat-denatured proteins). Thompson and Yielding have shown that the number of available ANS binding sites on glutamate dehydrogenase increases upon the addition of Zn^{+2}. The reagent is known to favor subunit dissociation and stimulate monocarboxylic L-amino acid dehydrogenase activity. (9) Less attention has been given to the study of actual binding processes for which fluorescence is also a powerful method. Recent work (5) has led to an understanding of the specificity with which a protein binds a ligand in terms of a site on the protein which may interact with the ligand through ionic forces, hydrogen bonds or hydrophobic forces.

The ability of many proteins to bind nonbiological "artificial probes", which are designed to mimic natural ligands or to bind to hydrophobic protein regions, has also been utilized in studying protein binding. The fluorescence

of these probes has been characterized in a large number of solvents and solvent mixtures. Generally, the transfer of the probe from a nonpolar to polar solvent results in a red shift of the fluorescence emission maximum, an increase in the band width, and a precipitous drop in the quantum yield. (5) As mentioned above, one of the probes which shows this effect is 1-anilino-8-napthalenesulfonate (ANS). This was shown in the study of the complex of ANS and aponyoglobin. Aponyoglobin, which is myoglobin minus its heme group, was chosen because it has a highly nonpolar heme-binding site. The inference that the fluorescence properties of ANS depend on the polarity of its environment was supported by studies of the emission of this compound in various organic solvents. (2) The emission spectra of ANS in a series of alcohols are shown in Figure 3. As the polarity of the solvent decreases, the quantum yield increases and the emission maximum shifts toward the blue. A similar effect of solvent polarity on quantum yield and emission maximum was observed for ANS in mixtures of ethanol and water (Fig. 4).

In the course of examining derivatives of 7-chloro-4-nitrobenzafurazan, NBD-Cl, II,

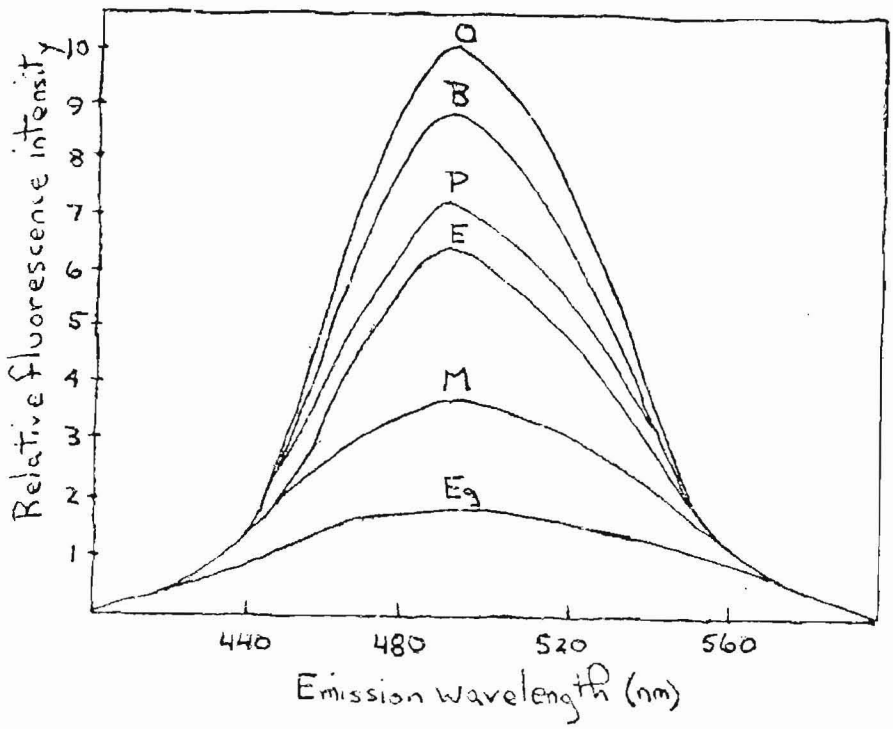


Figure 3. Fluorescence emission spectra of 1-anilino-8-naphthalene sulfate (ANS) in alcohols. The quantum yield increases and the emission maximum shifts as the solvent polarity decreases in the order: 1,2-ethanediol (Eg), methanol (M), ethanol (E), 1-propanol (P), 1-butanol (B), and 1-octanol (O). (Reference 2)

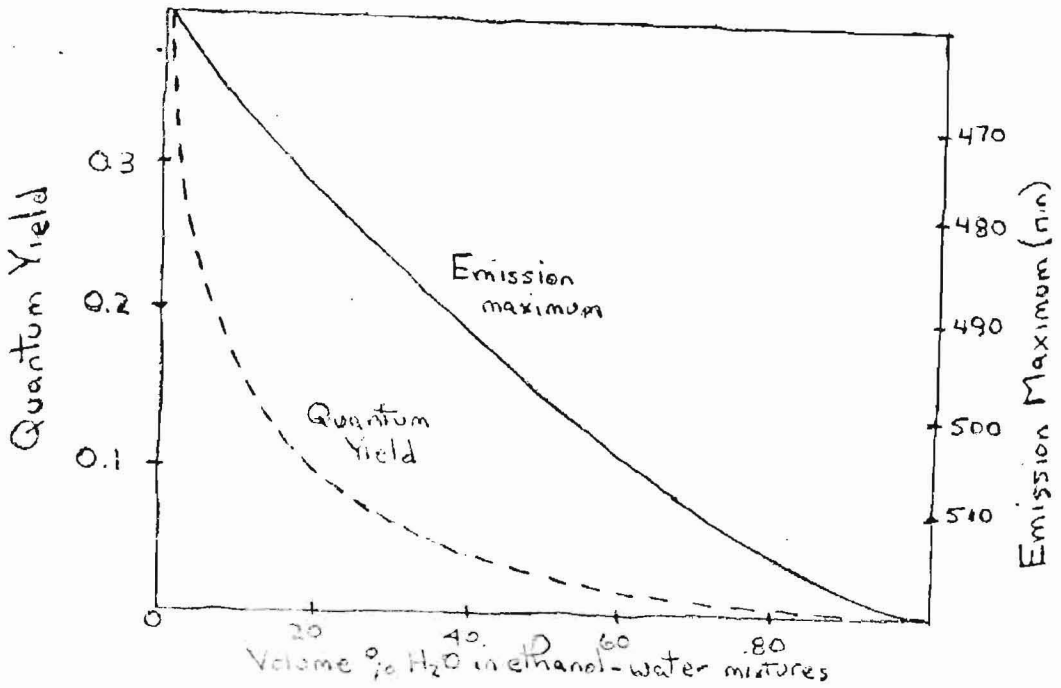
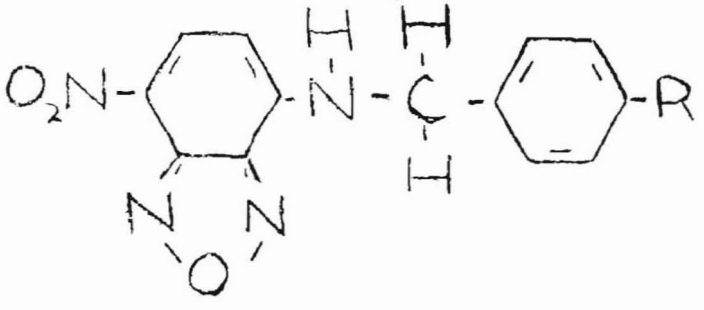


Figure 4. Dependence of quantum yield and emission maximum of ANS on the percent (by volume) of H_2O in H_2O-ethanol mixtures. (Reference 2)

for potential antileukemic activity, Ghosh and Whitehouse noticed that several 7-amino derivatives were highly fluorescent at low dilutions when the amino group was derived from an aliphatic amine. (10) The strong fluorescence of the probes is best observed in solvents of low polarity and when it is excited by visible light (464nm). Kenner and Aboderin describe the use of two specific derivatives of 7-chloro-4-nitrobenzofurazan, i.e., 7-benzylamino-4-nitrobenzofurazan (III), and 7-(p-methoxybenzylamino)-4-nitrobenzofurazan (IV).



III , R=H
IV , R=OCH$_3$
V , R=CH$_3$
VI , R=Cl

These compounds are sensitive fluorescent reporter groups of nonpolar areas and conformational changes in proteins and nucleoprotein particles. The compounds have been used to follow the conformational changes associated with the autoactivation of bovine trypsinogen. Also the binding of IV to the 50S ribosomal subunit of Escherichia coli was described and interpreted in terms of a unique hydrophobic pocket resulting from protein-nucleic acid interaction.

In this study, two new derivatives of the parent compound were made, 7-(p-methylbenzylamino)-4-nitrobenzofurazan (V) and 7-(p-chlorobenzylamino-4-nitrobenzofurazan (VI) and their fluorescence properties were studied. Attempts were made to prepare other p-derivatives of the parent compound, for example, p-NO_2, p-NH_2, p-OH, and p-COOH. Work was in progress on other compounds similar to these but results have not been published. (11,4)

EXPERIMENTAL METHODS

Ultraviolet and visible spectra were measured on a Beckman Model DK-2A Spectrophotometer. Fluorescence spectra were obtained using an Aminco-Bowman Spectrophotofluorometer (SPF). The nuclear magnetic resonance spectra were run on a Varian T-60A NMR with tetramethylsilane used as the internal standard. The solvent used with these compounds was deuterated-dimethylsulfoxide which gave a peak at 2.5 with the TMS peak at 0.0 (Fig. 5).

Materials

All starting compounds and solvents were reagent or spectroscopic grade.

Preparation of 4-Chloro-7-Nitro-Benzofurazan (11)

2,6-Dichloronitrosobenzene. A solution of 16.2 grams (0.10 moles) of 2,6-dichloroaniline in a mixture of glacial acetic acid (400 ml) and 30% hydrogen peroxide (80 ml, 0.70 moles) was allowed to stand at room temperature for 48 hours. Crystals were removed and recrystallized from glacial acetic acid to give 6.16 grams (0.034 moles, 26%) of product, mp 173°-175°C (lit. 175.5-176°C).

4-Chlorobenzofurazan. To 75 ml of dimethyl sulfoxide containing sodium azide (3.5 grams, 0.54 moles) was added 6.16 grams (0.034 moles) of 2,6-dichloronitrosobenzene; this

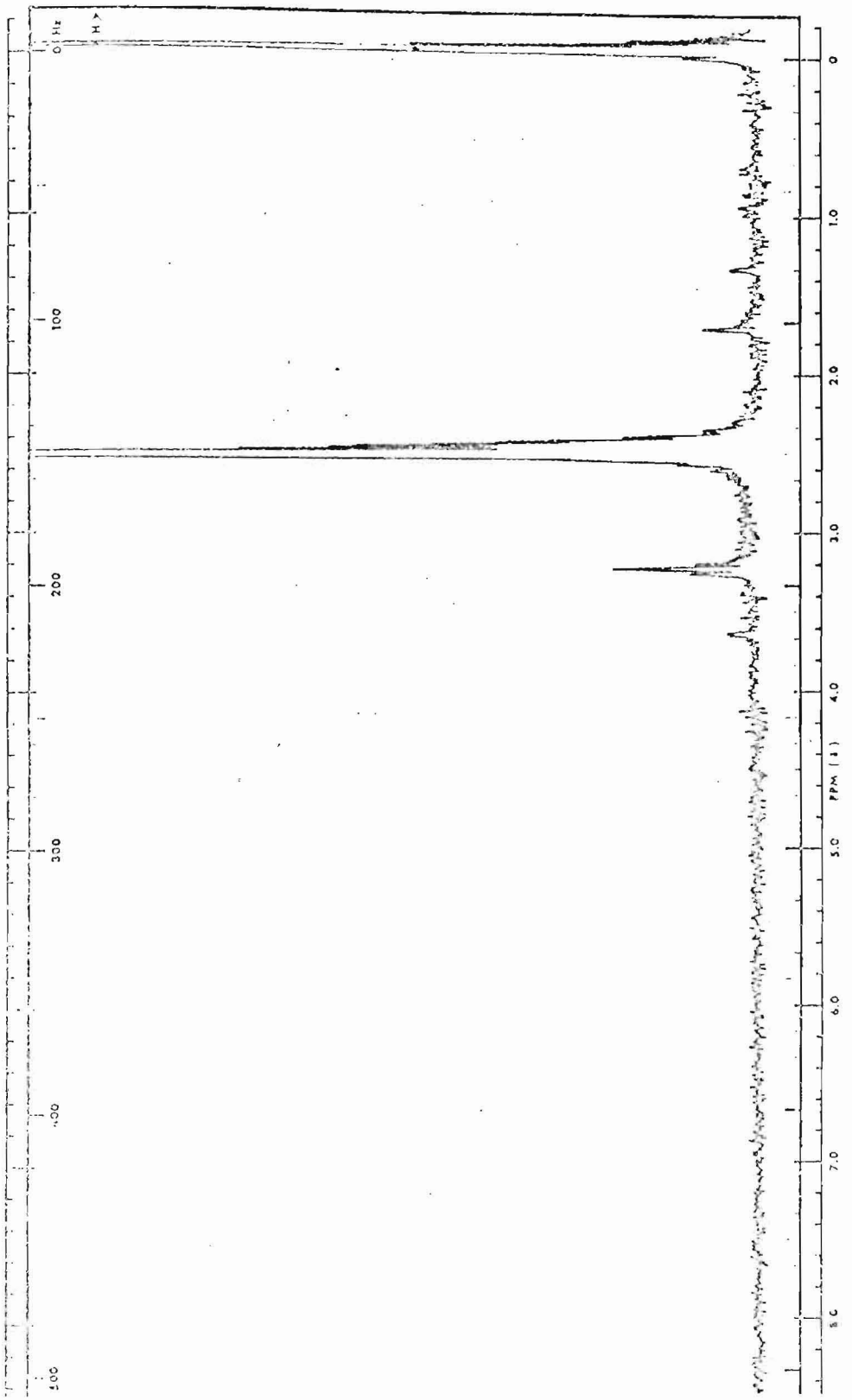


Figure 5. NMR spectrum of DMSO-d_6.

solution was then heated to 100°C and shaken. At a temperature of 50°C, the reaction mixture began to effervesce and cooling was necessary to moderate the reaction and to keep the temperature at 100°C. After the effervescence has ceased, the temperature was raised to 120°C for one minute. The solution was cooled and 100 ml of water was added to give a brown precipitate. The precipitate did not crystallize from aqueous ethanol as reported in the literature, the yield of the unpurified precipitate was 3.79 g (0.025 moles, 62%); mp 82-84°C (lit. 83-84°C).

4-Chloro-7-nitrobenzofurazan. In a 100 ml beaker, 3.78 grams (0.025 moles) of 4-chlorobenzofurazan was dissolved in 98% sulfuric acid (30 ml). To this solution 2.5 grams (0.03 moles) of sodium nitrate in 50% sulfuric acid (30 ml) was added dropwise with stirring to maintain a temperature of 60°C. After the addition was complete, the temperature was raised to 85°C for 30 minutes. The solution was then poured on 250 grams of crushed ice giving an orange solid. The orange solid was crystallized from aqueous ethanol to give pale yellow needles--4.90 grams (0.024 moles, 98%); mp 95-97°C (lit. 96.5-97°C).

7-Benzylamino-4-nitrobenzofurazan. (4) 7-Benzylamino-4-nitrobenzofurazan was synthesized by adding 0.50 grams (4.7 mmoles) of benzylamine to 0.80 grams (4.0 mmoles) of 4-chloro-7-nitrobenzofurazan, each in 80 ml of ethyl acetate at room temperature. After 2 hours of stirring,

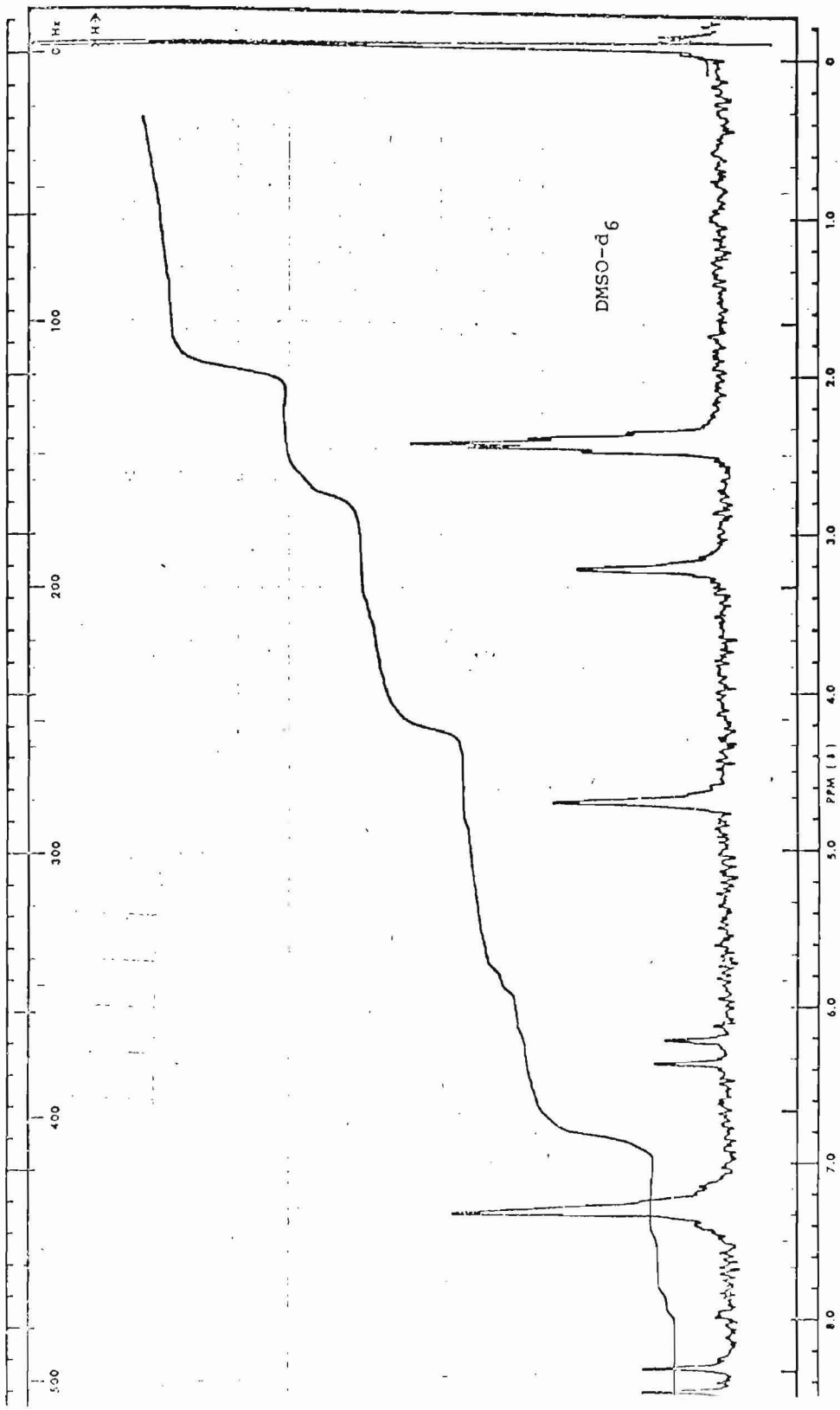


Figure 6. NMR spectrum of BBD.

15 ml of water was added and separated to remove excess amine. The organic layer was dried with anhydrous $MgSO_4$ and evaporated to dryness. Upon recrystallization from ethanol dark green crystals were obtained which yielded 0.092 grams (3.42 mmoles) of product (75.4%); mp 207-208°C (lit. 207-208.5°C). NMR(DMSO-$d_6$$Me_4$Si) δ-4.7(s,2H), 7.3(s,5H), 6.3(d,1H), 8.4(d,1H). (See Fig. 6.)

7-(p-Chlorobenzylamino)-4-nitrobenzofurazan. 7-(p-chlorobenzylamino-4-nitrobenzofurazan was synthesized by adding 0.51 grams (3.7 mmoles) of p-chlorobenzylamine to 0.79 grams (4.0 mmoles) of 7-chloro-4-nitrobenzofurazan each in 80 ml of ethyl acetate at room temperature. After 2 hours of stirring, 15 ml of water was added and separated to remove excess amine. The organic layer was dried with anhydrous $MgSO_4$ and evaporated to dryness. Upon recrystallization from ethanol dark green crystals were obtained, 0.109 grams (3.59 mmoles) (80.4%); mp 196-197°C (no lit. mp). NMR (DMSO-d_6; Me_4Si) δ-4.7(s,2H), 7.4(s,4H), 6.3(d,1H), 8.4(d,1H). (See Fig. 7.) Anal. Calcd* for $C_{12}H_9N_4O_3Cl$:C, 51.25; H, 2.98; Cl, 11.64. Found: C, 50.73; H, 2.89; Cl, 11.77.

7-(p-Methylbenzylamino)-4-nitrobenzofurazan. This compound was synthesized by adding 0.50 grams (4.1 mmoles) of p-methylbenzylamine with 0.71 grams (3.6 mmoles) of

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*Analysis were performed by Galbraith Laboratories, Inc., 2323 Sycamore Dr., Knoxville, Tennessee 37921--Sample No.-X-2575.

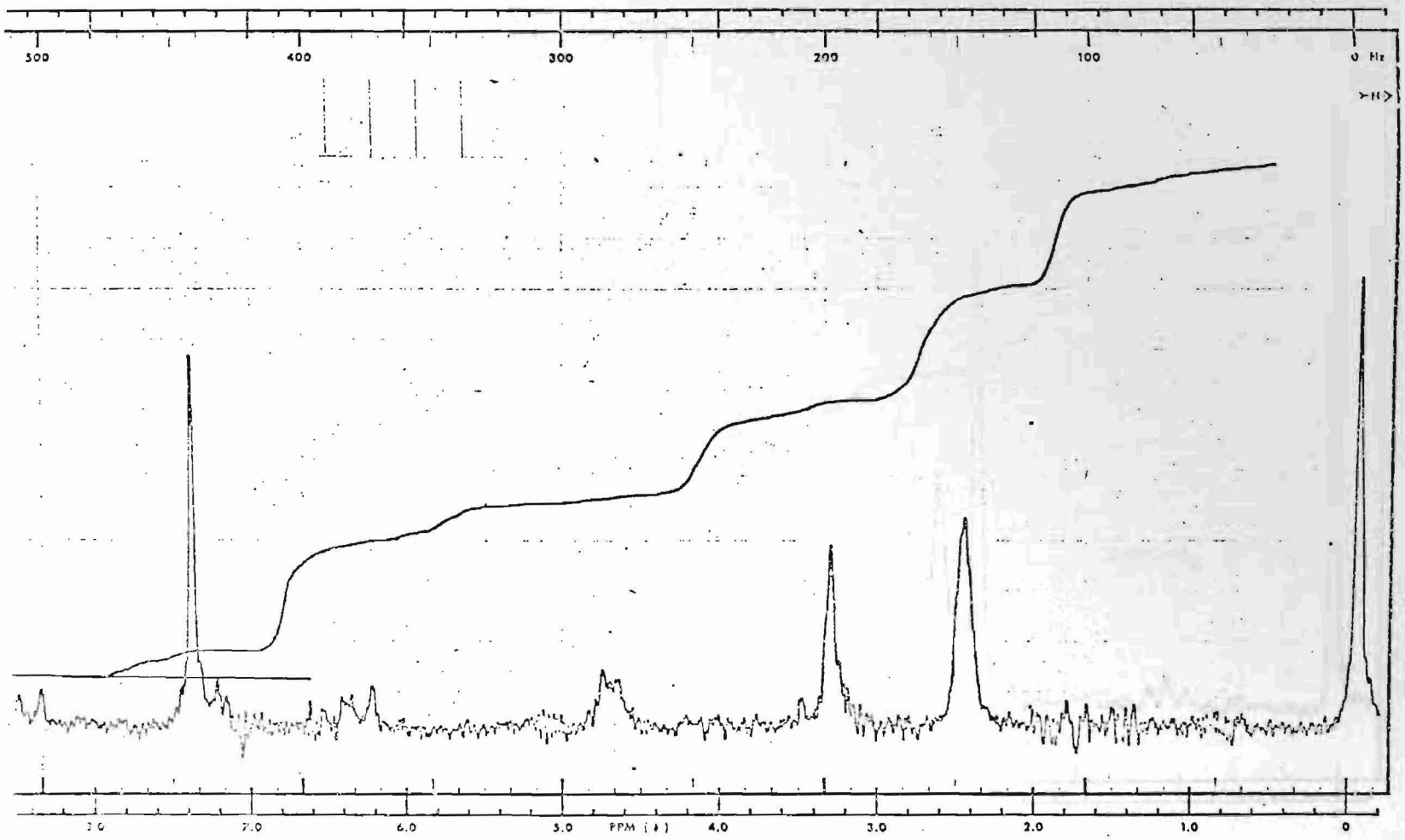


Figure 7. NMR spectrum of BBD-Cl.

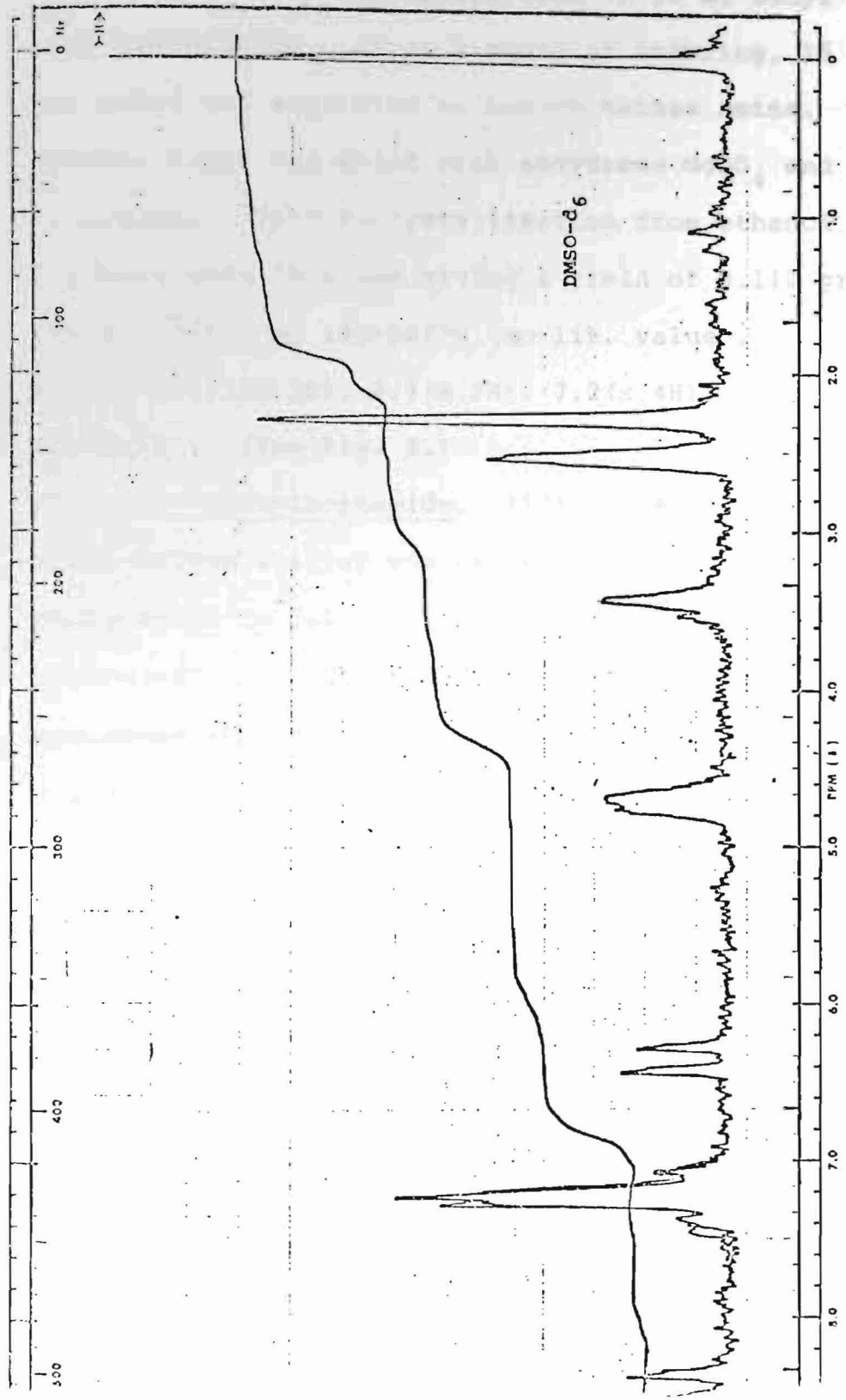


Figure 8. NMR spectrum of BBD-$\underline{CH_3}$.

7-chloro-4-nitrobenzofurazan each in 80 ml ethyl acetate at room temperature. After 2 hours of stirring, 15 ml of water was added and separated to remove excess amine. The organic layer was dried with anhydrous $MgSO_4$ and evaporated to dryness. Upon recrystallization from ethanol dark green crystals were obtained giving a yield of 0.112 grams (3.96 mmoles, 98%); mp 196-197°C (no lit. value). NMR (DMSO-d_6; Me_4Si) δ-2.2(d,3H), 4.7(s,2H), 7.2(s,4H), 6.4(d,1H), 8.4(d,1H). (See Fig. 8.)

N-Benzylacetamide. (12) In a 1-liter flask with a motor-driven stirrer was placed 108 grams (1 mole) of benzylamine in 300 ml of water. The mixture was stirred vigorously, then 215 grams (2 moles) of acetic anhydride was added in one portion. Vigorous stirring was continued for 25 minutes during which time the solution became hot. There were no crystals seen and the solution was placed in the refrigerator overnight. After two days in the refrigerator, no crystals were seen. The solution was placed on the vacuum evaporator and the water was removed. To this solution, 10% sodium hydroxide was added to raise the pH to 5.5. The solution was extracted with ether and the ether layer was evaporated to a small volume. With chilling, crystals formed. The crystals were re-crystallized from 95% ethanol and filtered, with yield 92.96 grams (0.6197 moles, 63%) (white needles), mp 55-56°C (lit. mp 60°C). (12)

p-Nitrobenzylacetamide. (12) A 250 ml three-necked flask was fitted with a Hershberg stirrer, a reflux condenser, and a dropping funnel. The flask was charged with 165 ml of acetic anhydride, and 28 grams (0.25 moles) of benzylamine was introduced from a dropping funnel. The mixture became very warm. After the amine had been added, the solution was cooled to 12-13°C in an ice-salt bath. During the cooling, the dropping funnel and condenser were replaced by another dropping funnel containing 32 ml of 70% nitric acid and a thermometer. The temperature was kept below 15°C and the nitric acid was added during a 1/2-hour period. The solution was stirred for another 1/2-hour. A yellow oil was recovered upon normal workup, all attempts to purify the crude p-nitrobenzylacetimide failed.

p-Nitro-benzylphthalimide. (13) Potassium phthalimide, 5.0 grams (0.03 moles) was added to a solution of 4.4 grams (0.03 moles) of p-nitrobenzyl chloride in 20 ml of dimethylformamide. The reaction was stirred and heat was applied to raise the temperature to 55°C. After heating for 30 minutes, the reaction was cooled to 25°C. After addition of 30 ml of chloroform, the mixture was poured into 100 ml of water. The aqueous phase was separated and extracted with chloroform, the combined chloroform solutions were washed with 0.2 N sodium hydroxide to remove unreacted phthalimide. The chloroform layer was washed with water, dried with sodium sulfate, and the chloroform was evaporated.

The compound recovered had a mp 65-73°C (lit. value for α-chloro-p-nitrotoluene - 71°C) and was probably the starting material.

p-Hydroxybenzylamine. (13) An intimate mixture of 1.66 grams of anhydrous K_2CO_3 and 2.94 grams (0.020 moles) of phthalimide was treated with 5.00 grams (0.030 moles) of α-chloro-p-nitrotoluene and the mixture was heated in an oil bath at 190°C under a reflux condenser for 2 1/2 hours. The mixture was cooled and washed with water. After drying, the mass was ground and washed first with water and then aqueous ethanol, and filtered.

A solution of 4.5 grams of powdered $SnCl_2 \cdot 2H_2O$ in 6.0 ml of conc HCl was cooled and stirred in an ice bath. When the temperature of the solution had fallen to 5°C, the ice bath was removed, and 1.0 gram of the p-nitrotoluene phthalimide was added in one portion. The temperature rose slowly and heat was applied to raise it to 75°C. The solution was cooled for 2 hours and filtered. A suspension of the material in 60 ml of conc HCl was stirred and cooled to 4-5°C in an ice bath. A solution of 2 ml of 0.54 M $NaNO_2$ was added using a dropping funnel with the stem below the surface. As the solution was added, gas was given off. The crystals were filtered after an hour of cooling.

The wet crystals were added in small portion to 25 ml of boiling water. The solution was treated with Norit, filtered, and cooled. After two days of cooling, no product

was isolated.

α-Bromo-p-toluic acid. (14) A two-necked 25 ml reaction flask was equipped with a reflux condenser and a magnetic stirrer. To the flask was added 1.50 grams (0.11 moles) of p-toluic acid, 1.79 grams (0.10 moles) of N-bromosuccinimide, 0.029 grams (0.001 moles) of dibenzoyl peroxide and 55 ml of carbon tetrachloride that had been dried over calcium chloride. The reaction was flushed with dry nitrogen and a drying tube was attached to the reflux condenser. The stirrer was turned on and the reaction was heated to reflux temperature with an oil bath for one hour. The reaction was allowed to cool and filtered to remove the succinimide, the filter was washed twice with 2 ml portions of dry carbon tetrachloride. The carbon tetrachloride was removed by evaporation. The product was not isolated.

Determination of Quantum Yield (5)

Quantum efficiencies (Quantum yield, q) were evaluated by the use of the relationship:

$$F = I_E q \% A G(\theta)$$

where F = observed fluorescence of dye solution,

G(θ) = geometry factor (<1) since not all of the fluorescent light is observed,

I = intensity of exciting light,

q = quantum yield,

%A = percent absorption of solution (100-%T).

The ratio of the quantum yield of the standard dye (qs) and the unknown dye (qx) is:

$$\frac{Fx}{Fs} = \frac{qx}{qs}$$

where Fx is the measured fluorescence of the unknown and Fs is that of the standard. In order for this to be true, the ratio shown must be equal to unity. The best way to insure this is to excite both standard and sample at the same wavelength and to have the solutions of equal absorbancy at this wavelength:

$$\frac{I_{Ex}\%Ax}{I_{Es}\%As} = 1$$

Since a fluorescent screen detector system was used, no corrections were needed to be made for the instrument response since the response is directly proportional to the total fluorescence intensity spectrum. By comparing the peak fluorescence intensities of the unknown dye with that of the peak fluorescence intensities of the reference dye, the quantum yield determinations could be calculated.

The known compound used in the fluorescence part of this study was quinine bisulfate with a quantum yield of 0.55 in 1.0 N sulfuric acid. (5)

Preparation of Solutions for Absorption and Fluorescent Spectra

The solutions that were used for spectrophotometric analysis were made on the same day as the analysis. The

absolute ethanol used was checked using a pyconmeter, giving a specific gravity of 0.798 (0.798-lit. value of 100% ethanol). (17) The dimethylformamide was dried over molecular sieves and phosphorus pentoxide. The benzofurazan derivative was weighed on a Sartorius Micro-Balance and dissolved in the solvent being used. The solution was then transferred to a 100 ml volumetric flask and diluted to the mark. A typical stock solution was 0.0042 grams of BBD dissolved in 100% ethanol and transferred to a 100 ml volumetric flask, cf. Table 1. When the spectrophotometric analysis was done, dilutions of this solution was made for the various concentrations of compound. The various concentrations of ethanol solutions were made by diluting the stock solution with ethanol and distilled water.

Table 1

Stock Solutions of the Compounds

BBD:	0.0042g/100 ml of 100% ethanol
BBD-CH_3:	0.0049g/100 ml of 100% ethanol
BBD-Cl:	0.0045 g/100 ml of 100% ethanol
BBD:	0.0020g/100 ml of 100% dimethylformamide
BBD-CH_3:	0.0021g/100 ml of 100% dimethylformamide
BBD-Cl:	0.0021g/100 ml of 100% dimethylformamide

The dimethylformamide solutions were prepared in a similar manner. These solutions were run on the spectrophotometric instruments at the concentrations shown in Table 2.

Table 2

Solution Concentrations for Absorption and Fluorescent Spectra

	Conc. of ethanol	Conc. of Compound (M)
BBD	100%	1.5×10^{-5}
	80%	2.6×10^{-5}
	60%	1.9×10^{-5}
	40%	1.3×10^{-5}
	20%	0.7×10^{-5}
BBD-Cl	100%	2.6×10^{-5}
	80%	2.4×10^{-5}
	60%	1.8×10^{-5}
	40%	1.2×10^{-5}
	20%	0.6×10^{-5}
BBD-CH_3	100%	2.7×10^{-5}
	80%	2.9×10^{-5}
	60%	2.1×10^{-5}
	40%	1.4×10^{-5}
	20%	0.7×10^{-5}
	Conc. of DMF	Conc. of Compound (M)
BBD	100%	3.7×10^{-5}
	50%	3.7×10^{-5}
BBD-CH_3	100%	3.6×10^{-5}
	50%	3.6×10^{-5}
BBD-Cl	100%	3.1×10^{-5}
	50%	3.1×10^{-5}

RESULTS AND DISCUSSION

Absorption Spectra

The absorption spectra of 7-benzylamino-4-nitrobenzofurazan (BBD), 7-(p-methylbenzylamino)-4-nitrobenzofurazan ($BBDCH_3$) and 7-(p-chlorobenzylamino)-4-nitrobenzofurazan (BBD-Cl) in ethanol-water mixtures and dimethylformamide-water mixtures are shown in Figures 9-14 and summarized in Tables 3-5.

There was a shift in the absorption band of the $BBD-CH_3$ and BBD-Cl compounds as the ethanol-water mixtures were varied from 80% to 20%. Both $BBD-CH_3$ and BBD-Cl shifted in wavelength as the solvent system became more polar. The shift was toward the blue which is different from ANS. The molar absorptivities of BBD (ε = 17,900 M^{-1} cm) in 95% ethanol at 462 nm is close to the value reported by Kenner and Aboderin (19,700 M^{-1} cm^{-1}). (4) In Kenner and Aboderin's paper, they did not report if 95% ethanol was used; that could account for the different molar absorptivities of BBD. There were similar spectral shifts in the dimethylformamide solutions. These spectral results are similar to those obtained by Kenner and Aboderin. Unlike their results, the absorptivity in the 330=350 nm band appears to be different for all three compounds in all concentrations of ethanol, as shown in Table 4.

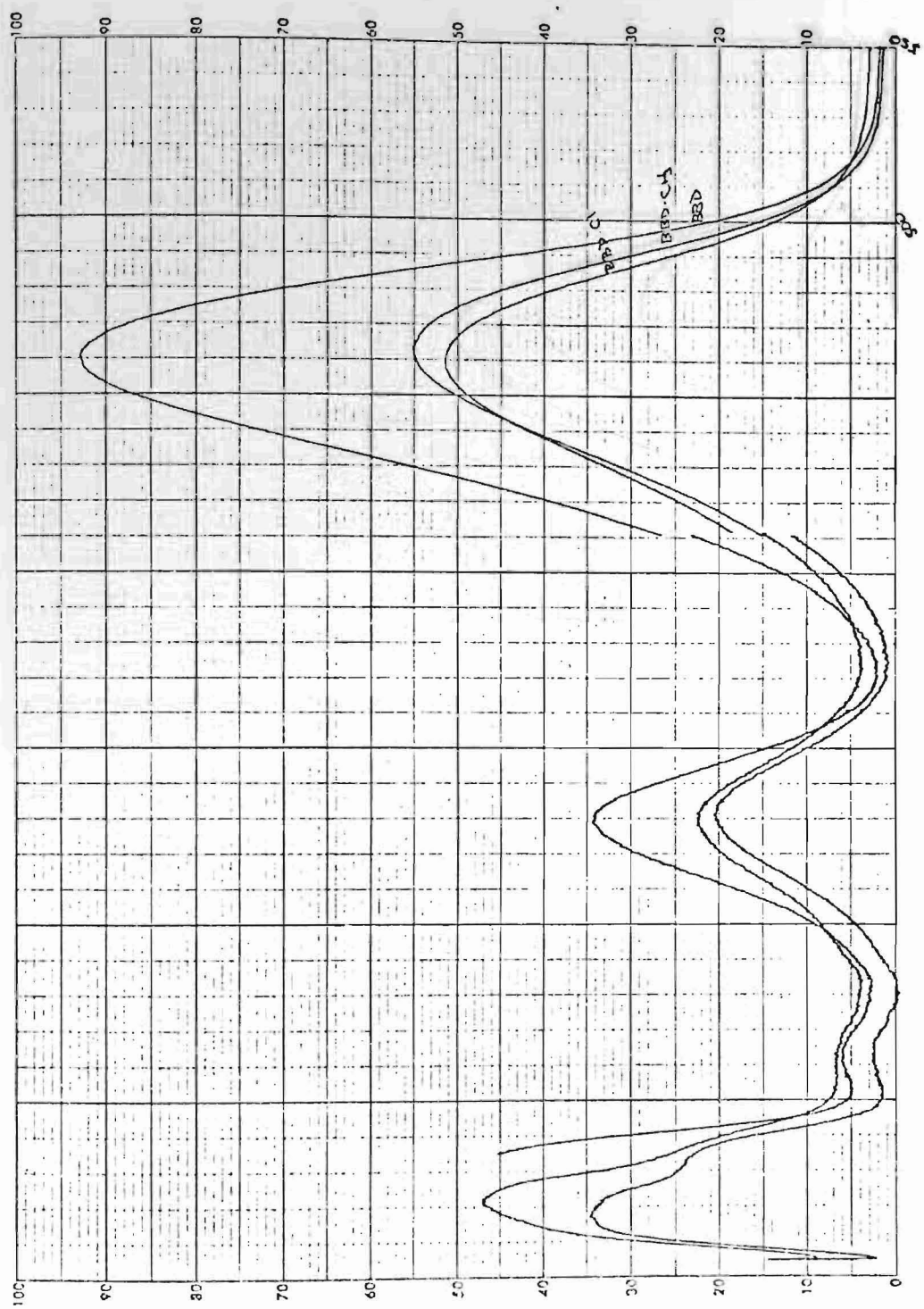


Figure 9. Visible spectra of BBD, BBD-CH_3, and BBD-Cl in 100% ethanol.

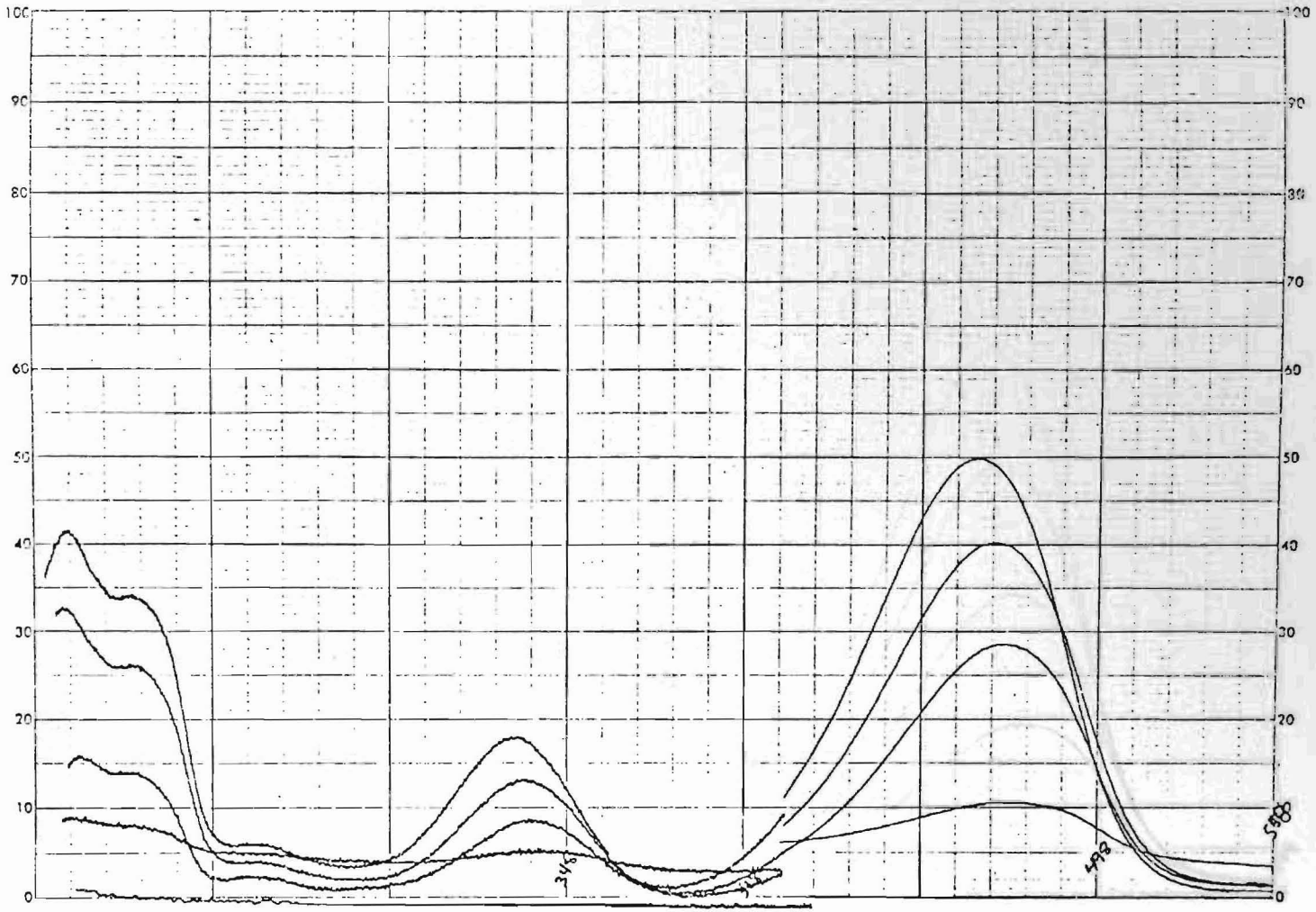


Figure 10. Visible spectra of BBD in ethanol-water mixtures.

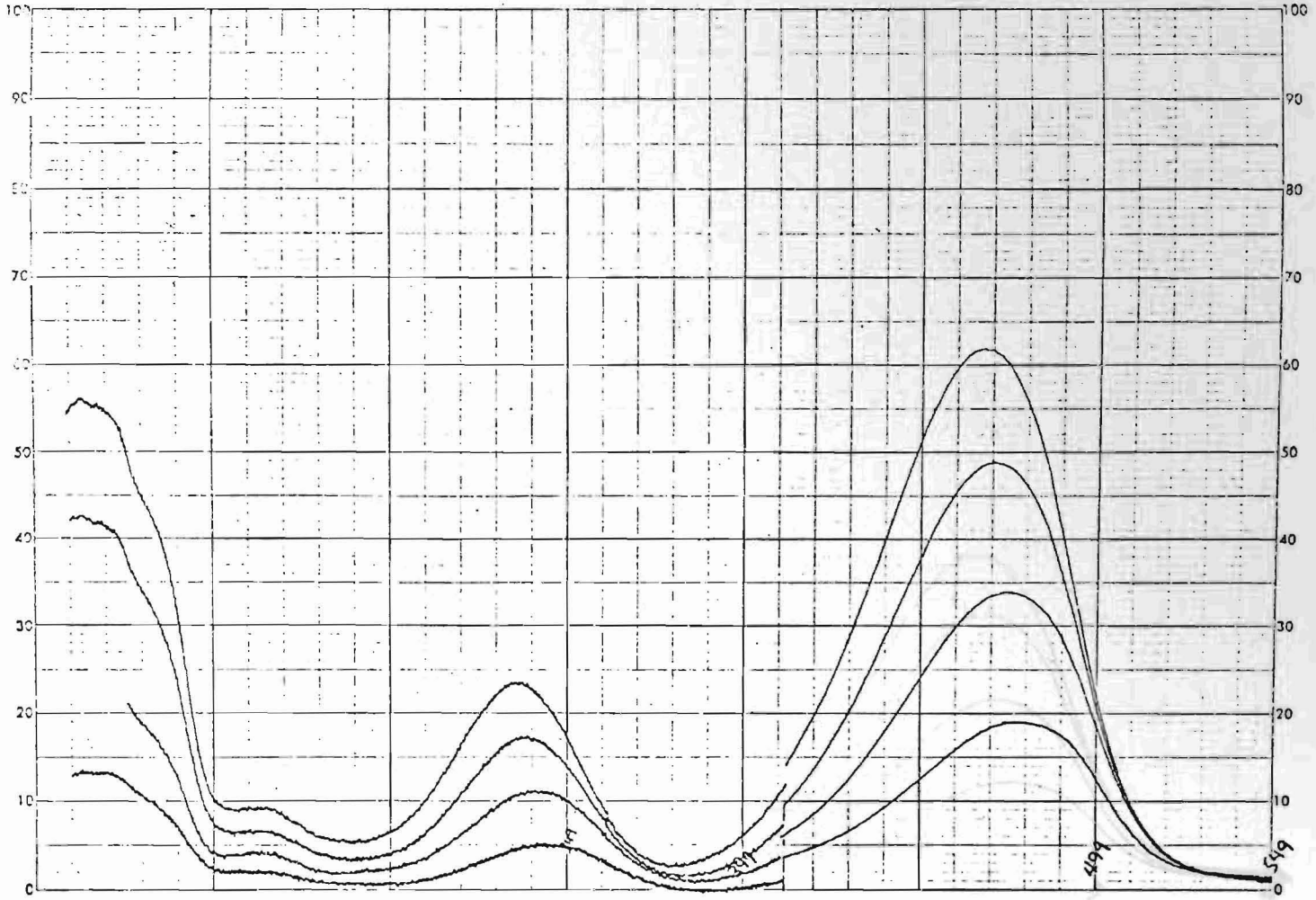


Figure 11. Visible spectra of BBD-CH_3 in ethanol-water mixtures.

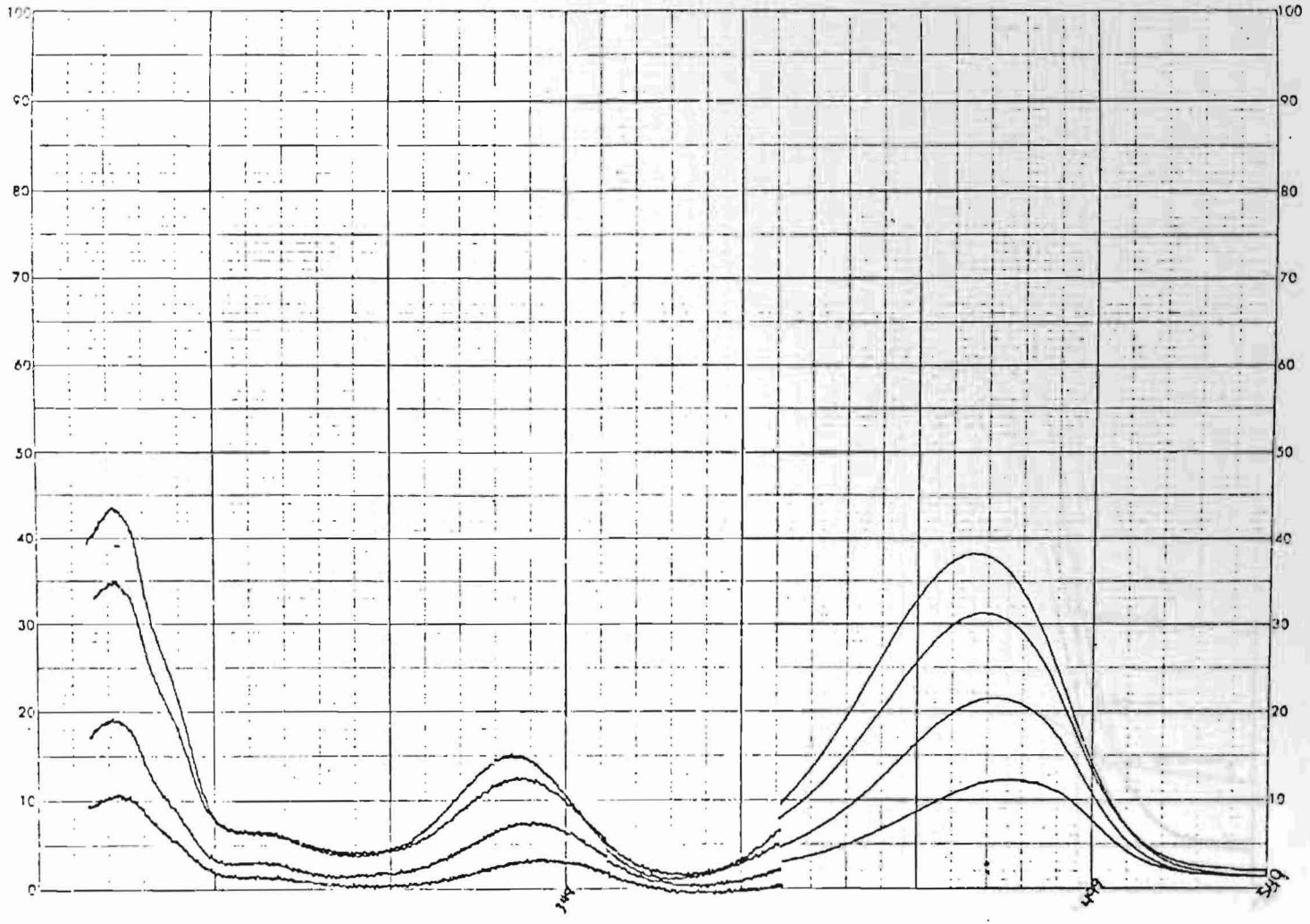


Figure 12. Visible spectra of BBD-Cl in ethanol-water mixtures.

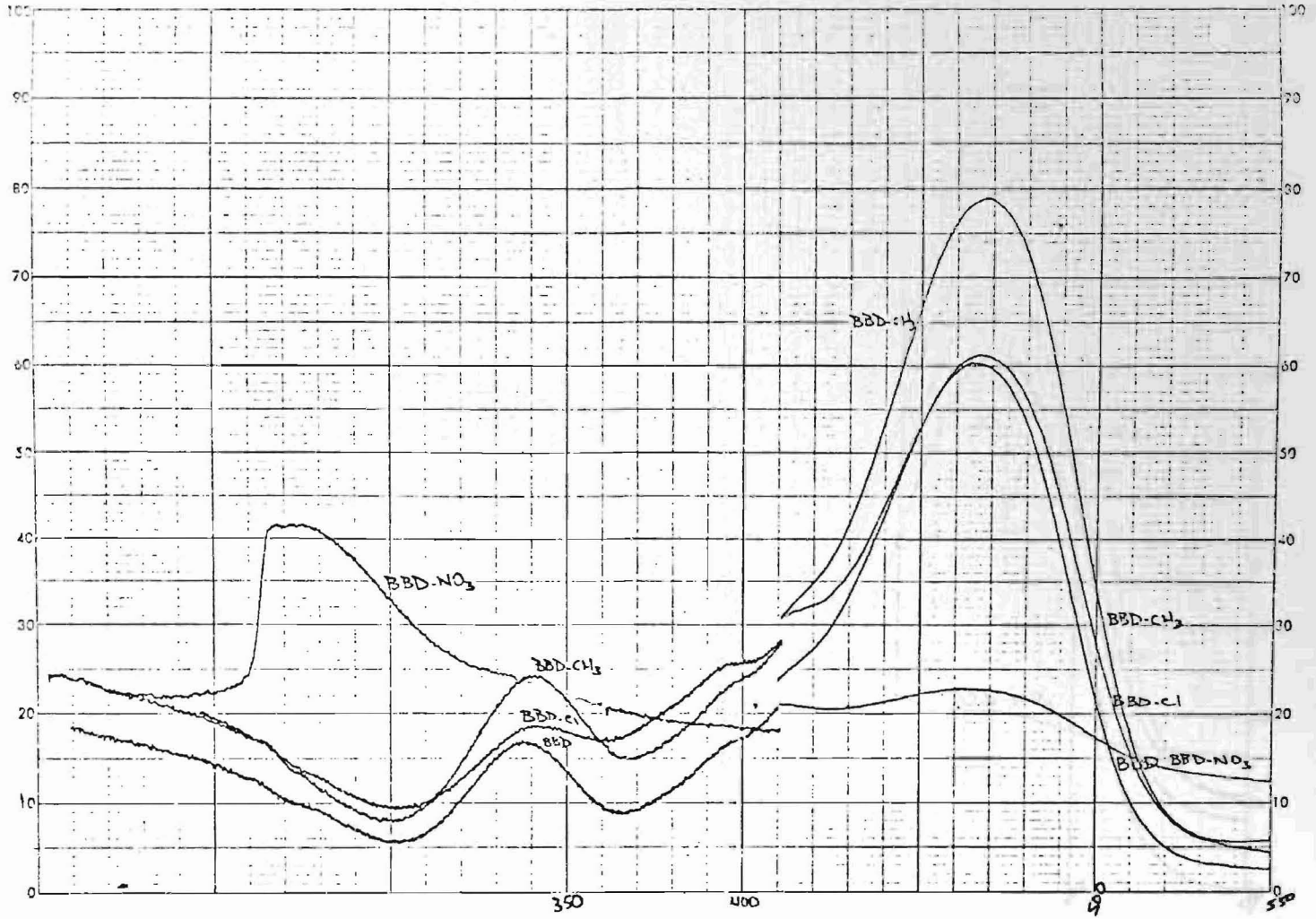


Figure 13. Visible spectra of BBD, BBD-CH_3, and BBD-Cl in 50% dimethylformamide solution.

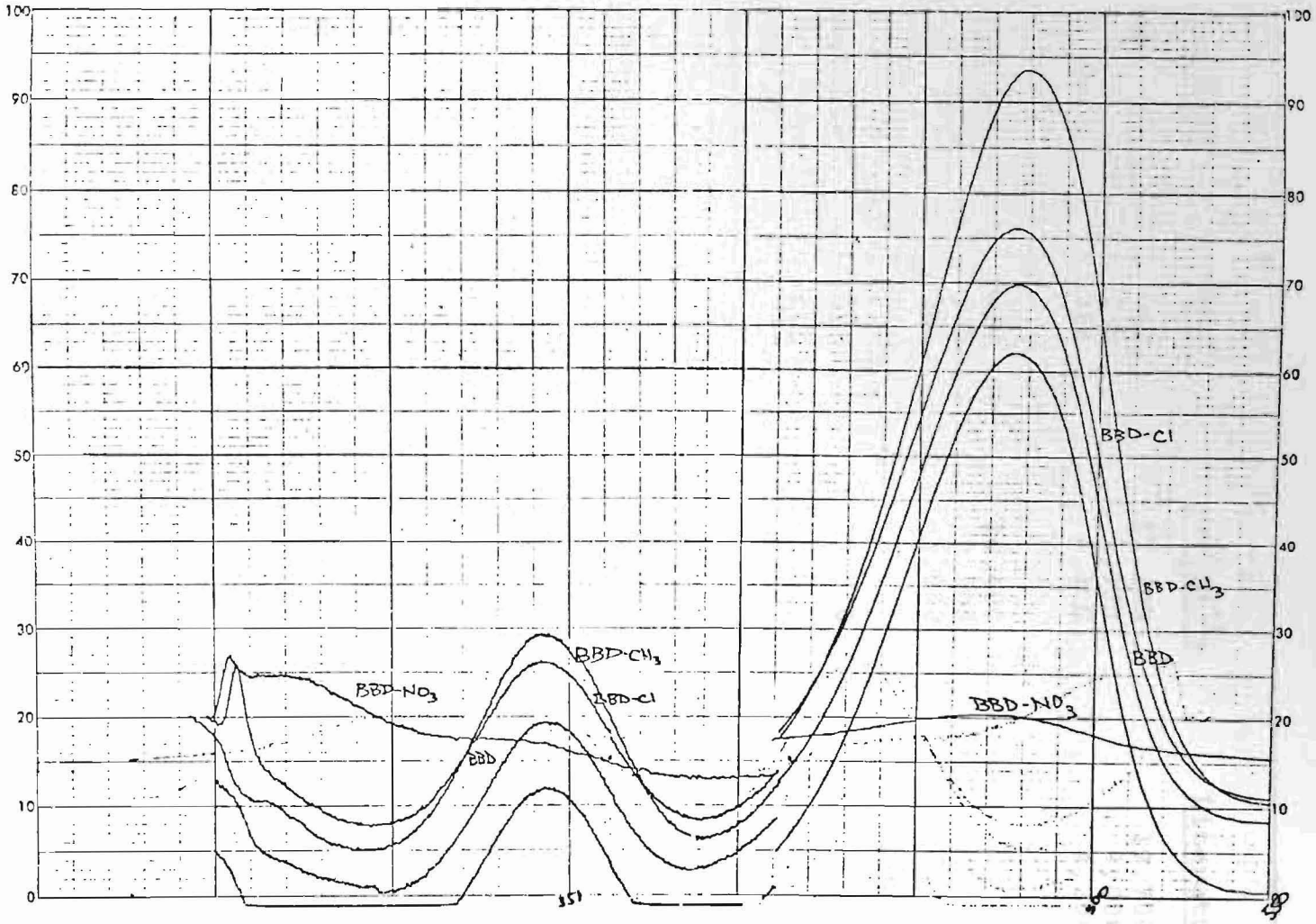


Figure 14. Excitation and emission spectra of BBD, BBD-CH_3, and BBD-Cl in 100% ethanol.

Table 3

U.V. Spectral Data of Compounds in 95% Ethanol

Compound	λ (nm)	$^{\circ}\varepsilon$ ($M^{-1}CM^{-1}$)	Literature[a]
BBD	462	17,900	19,700
	330	7,000	8,000
	264	4,400	4,200
BBD-CH_3	464	20,400	
	330	9,200	
	264	3,000	
BBD-Cl	460	19,500	
	330	5,700	
	262	3,800	

[a]Reference 4.

Table 4

Absorption Properties of BBD, BBD-CH_3, and BBD-Cl in Ethanol-Water Mixtures

% Ethanol (v/v)	BBD		BBD-CH_3		BBD-Cl	
	λ (nm)	ε (M^{-1} cm^{-1})	λ (nm)	ε (M^{-1} cm^{-1})	λ (nm)	ε (M^{-1} cm^{-1})
20%	264	3,100	265	3,500	265	4,200
	330	7,500	344	8,400	342	7,400
	462	23,100	478	27,000	473	17,600
40%	264	1,500	264	5,000	265	5,000
	330	6,500	342	10,000	338	8,300
	462	22,000	475	24,200	471	17,900
60%	264	2,100	264	4,500	262	5,000
	330	6,800	338	9,500	335	8,300
	462	21,000	470	23,300	467	17,200
80%	264	2,300	264	4,100	262	3,700
	330	7,000	335	9,000	332	7,500
	462	19,200	467	21,000	466	15,800

Table 5

Absorption Properties of BBD, BBD-CH_3, and BBD-Cl in Dimethylformamide

% DMF (v/v)	BBD		BBD-CH_3		BBD-Cl	
	λ (nm)	ε (M^{-1} CM^{-1})	λ (nm)	ε (M^{-1} CM^{-1})	λ (nm)	ε (M^{-1} CM^{-1})
50%	344	5,900	343	8,800	343	9,300
	465	18,900	478	25,000	465	24,500
100%	337	5,300	340	4,700	341	7,000
	464	16,200	468	21,900	466	19,700

The molar absorptivity of BBD-CH_3 in 20% ethanol (v/v) is about 14% greater at its absorption maximum at 478 nm than BBD at its corresponding maximum at 462 nm, and about 35% greater than BBD-Cl at its corresponding maximum at 473 nm.

The absorptivity in dimethylformamide was the same for BBD as the concentration of water was changed but it varied for the other two compounds, BBD-CH_3 and BBD-Cl. The BBD-CH_3 showed a much larger increase in absorptivity (ε = 8,800 M^{-1} cm in 50% DMF) relative to the other compounds.

Fluorescence Spectra

The three compounds show strong fluorescence in the range of 526 nm-536 nm when excited with light in the 460 nm region. Similar to the absorption spectra, BBD, BBD-CH_3, and BBD-Cl were run in ethanol-water mixtures; spectra are shown in Figures 15-20 and summarized in Tables 6 and 7. In using the peak fluorescence intensities to calculate the quantum yields of the compounds, it had to be shown that it gave the same results as using the integrated fluorescence peak areas. A quantum yield of 0.379 for BBD in 100% ethanol was calculated using the integration method with quinine sulfate as the reference dye. In this study, a quantum yield of 0.392 for BBD in 100% ethanol was found using the peak intensities.

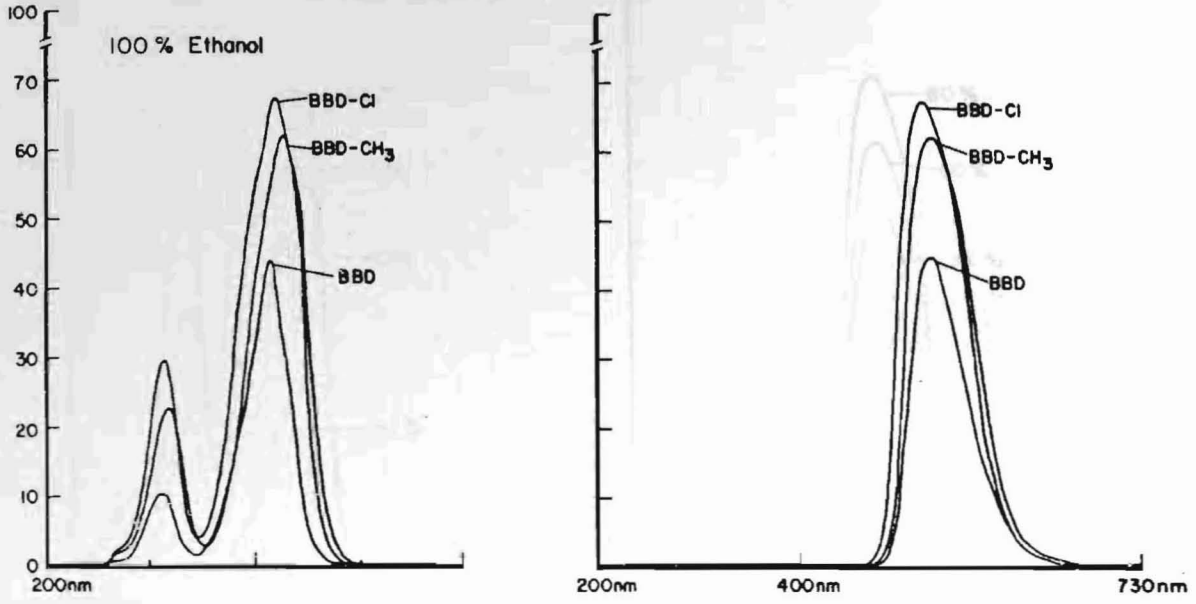


Figure 15. Excitation and emission spectra of BBD in ethanol-water mixtures.

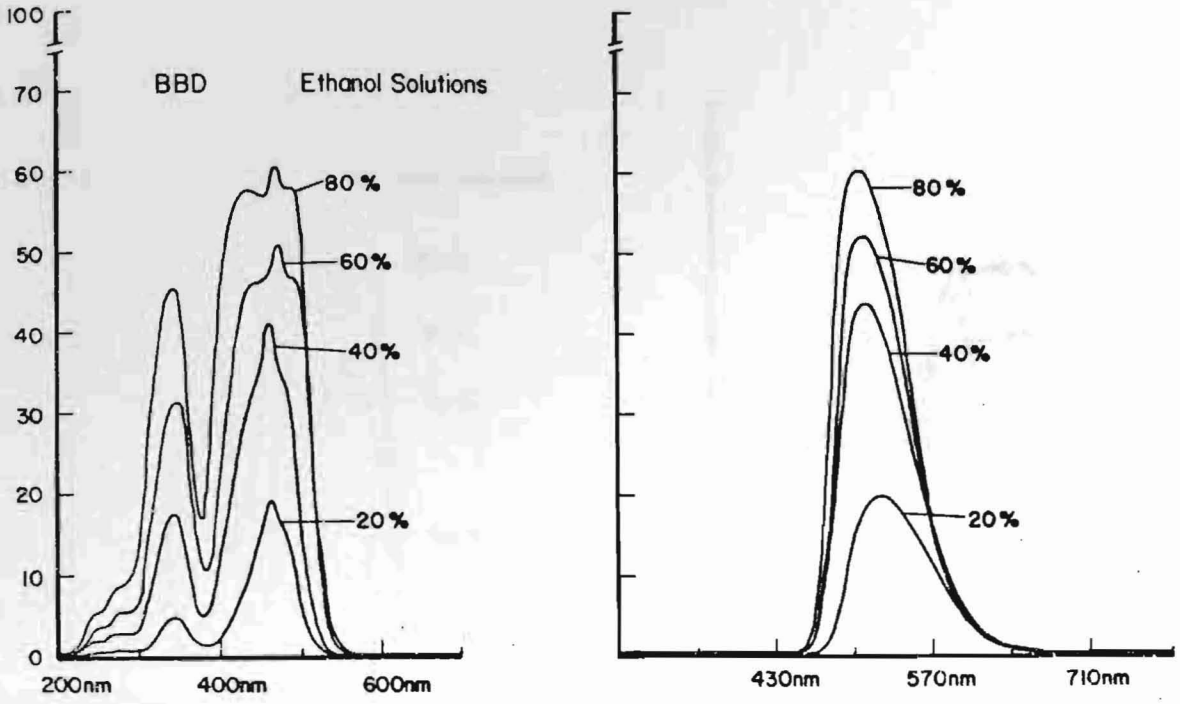


Figure 16. Excitation and emission spectra of BBD-CH_3 in ethanol-water mixtures.

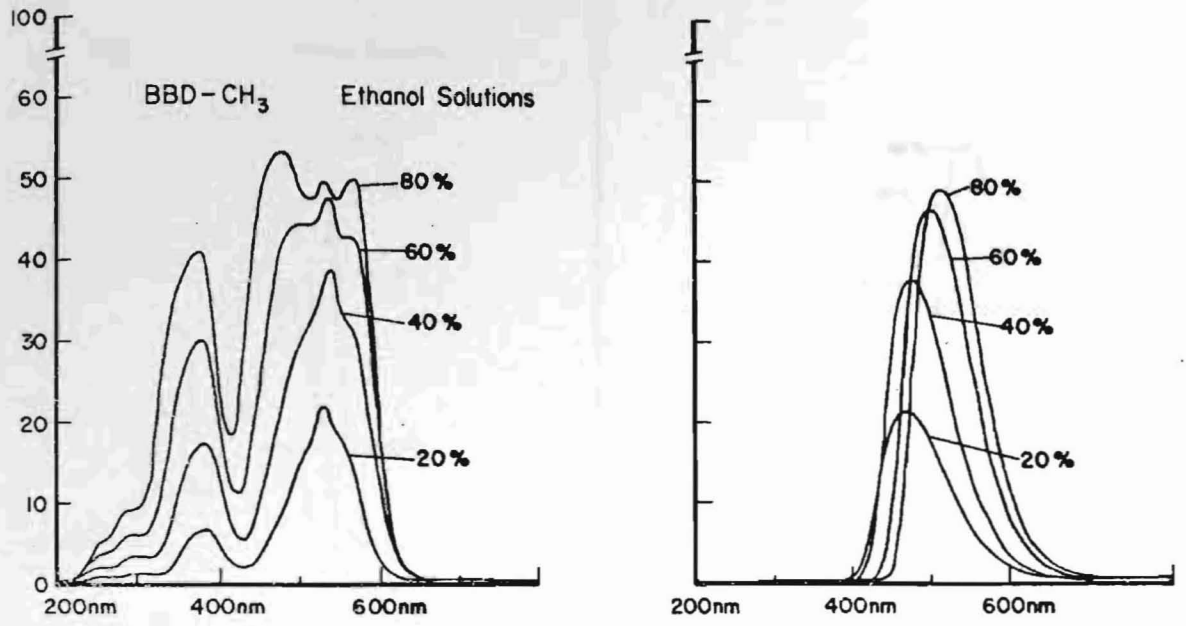


Figure 17. Excitation and emission spectra of BBD-Cl in ethanol-water mixtures.

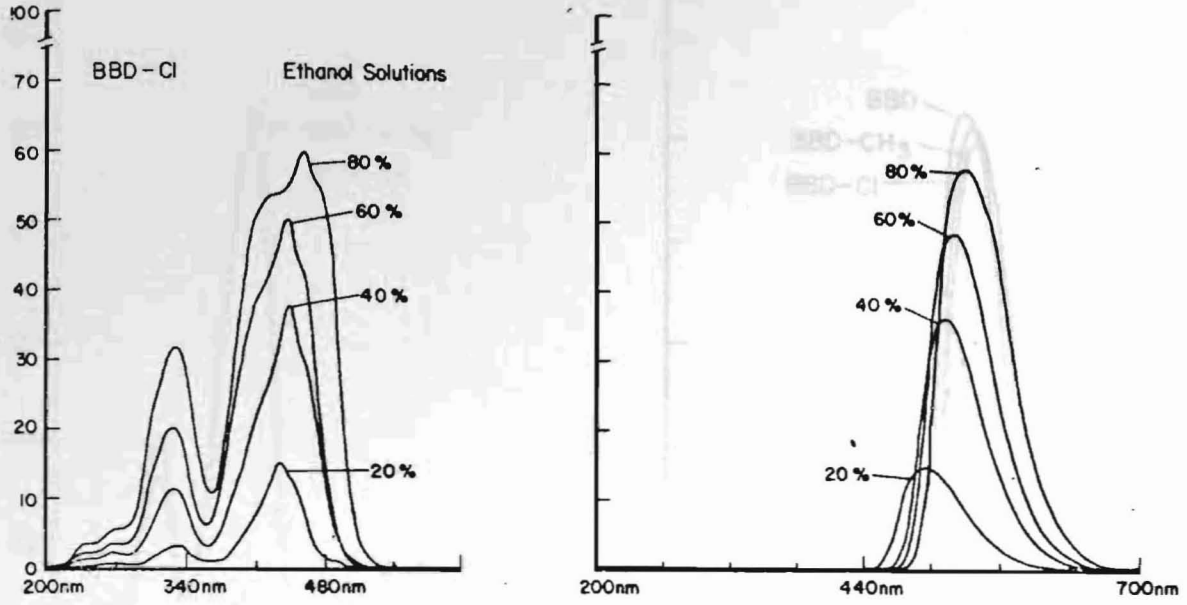


Figure 18. Excitation and emission spectra of BBD, BBD-CH_3, and BBD-Cl in 100% dimethylformamide.

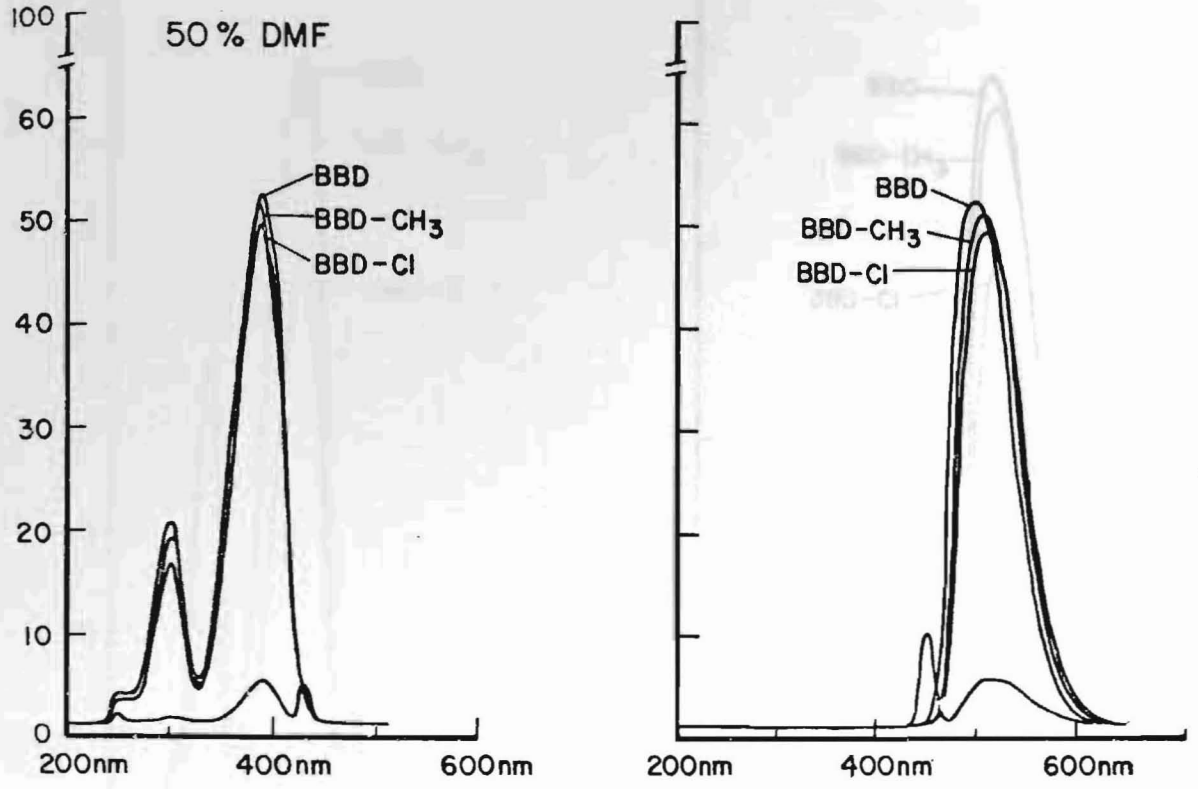


Figure 19. Excitation and emission spectra of BBD, BBD-CH_3, and BBD-Cl in 50% dimethylformamide.

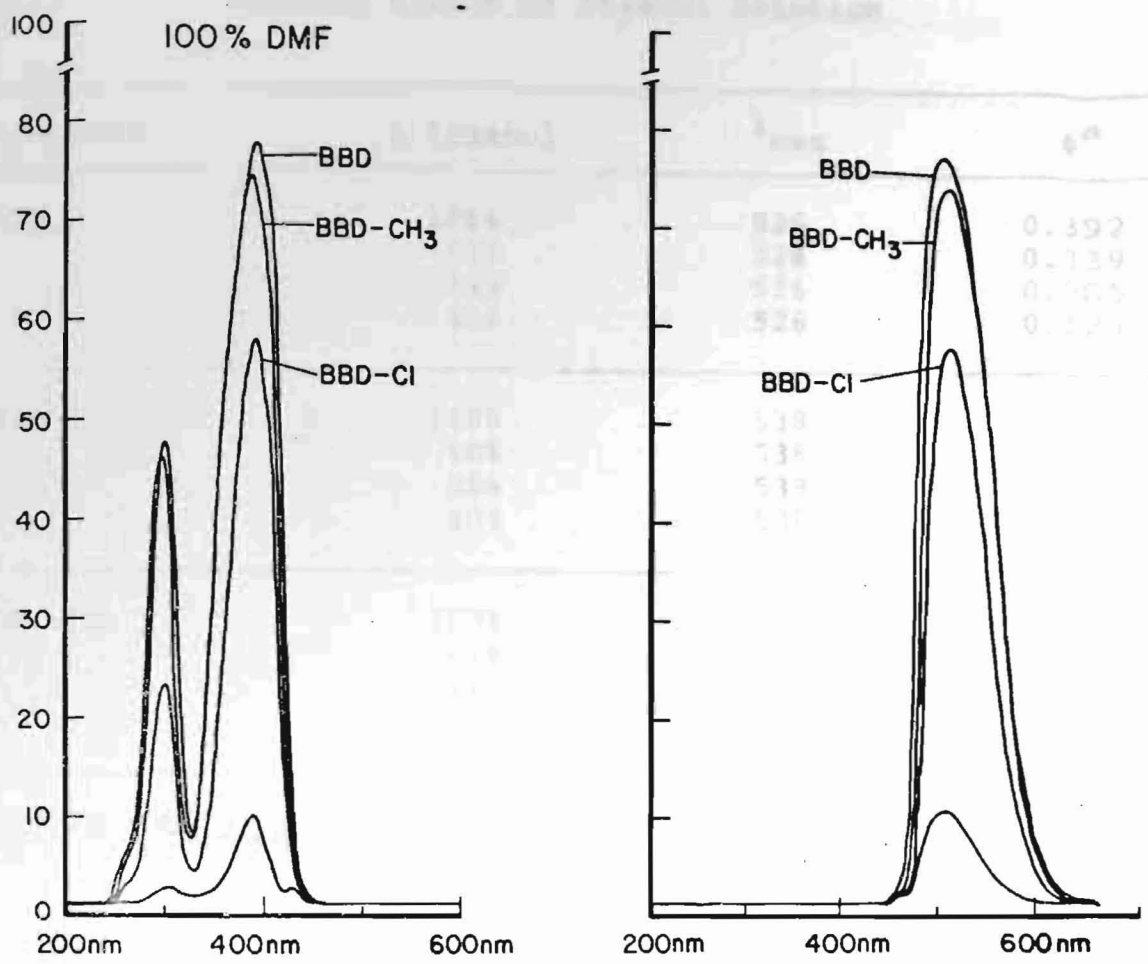


Figure 20. Visible spectra of BBD, BBD-CH_3, and BBD-Cl in dimethylformamide.

Table 6

Quantum Yields in Ethanol Solution

Compound	% Ethanol	λ_{max}	Φ^a
BBD	100%	526	0.392
	80%	526	0.339
	60%	526	0.285
	40%	526	0.129
BBD-CH_3	100%	538	0.317
	80%	538	0.301
	60%	538	0.246
	40%	538	0.137
BBD-Cl	100%	536	0.379
	80%	536	0.317
	60%	536	0.238
	40%	536	0.097

[a]Quantum yield

Table 7

Quantum Yields in Dimethylformamide Solutions

Compound	% DMF	λ_{max}	Φ
BBD	100%	526	0.504
	50%	526	0.343
BBD-CH_3	100%	530	0.482
	50%	530	0.336
BBD-Cl	100%	530	0.375
	50%	530	0.326

The quantum yields of these compounds became lower in the ethanol-water and dimethylformamide-water mixtures as the water concentration increased. The emission maxima of BBD reported by Kenner and Aboderin is different from the emission maxima found in this study but Kenner and Aboderin do not state the purity of their ethanol. The highest quantum yields were shown in 100% DMF except for BBD-Cl which showed similar quantum yields in both ethanol and dimethylformamide.

The potential usefulness of these compounds as fluorescent probes is illustrated by the results shown in Figure 21. The observed changes in quantum yield and wavelength of maximum emission with changes in solvent composition are reminiscent of the dependence of 2-p-toluidinylnaphthalene-6-sulfonate (Edelman and McClure) (6) and of dansylaminotyrosine derivatives (Kenner) (3) on solvent polarity. The quantum yields dropped as the solvent changed from ethanol to water. BBD and BBD-Cl showed the most sensitivity to solvent polarity. The quantum yields for both compounds decreased with increased solvent polarity, BBD-Cl showed the largest change, dropping from a quantum yield of 0.379 in 100% ethanol to 0.097 in 40% ethanol.

NMR Spectra

The NMR spectra of the analogs of 7-(benzylamino)-4-nitrobenzofurazan gave a fingerprint identification of these compounds. The spectra were integrated and the peaks

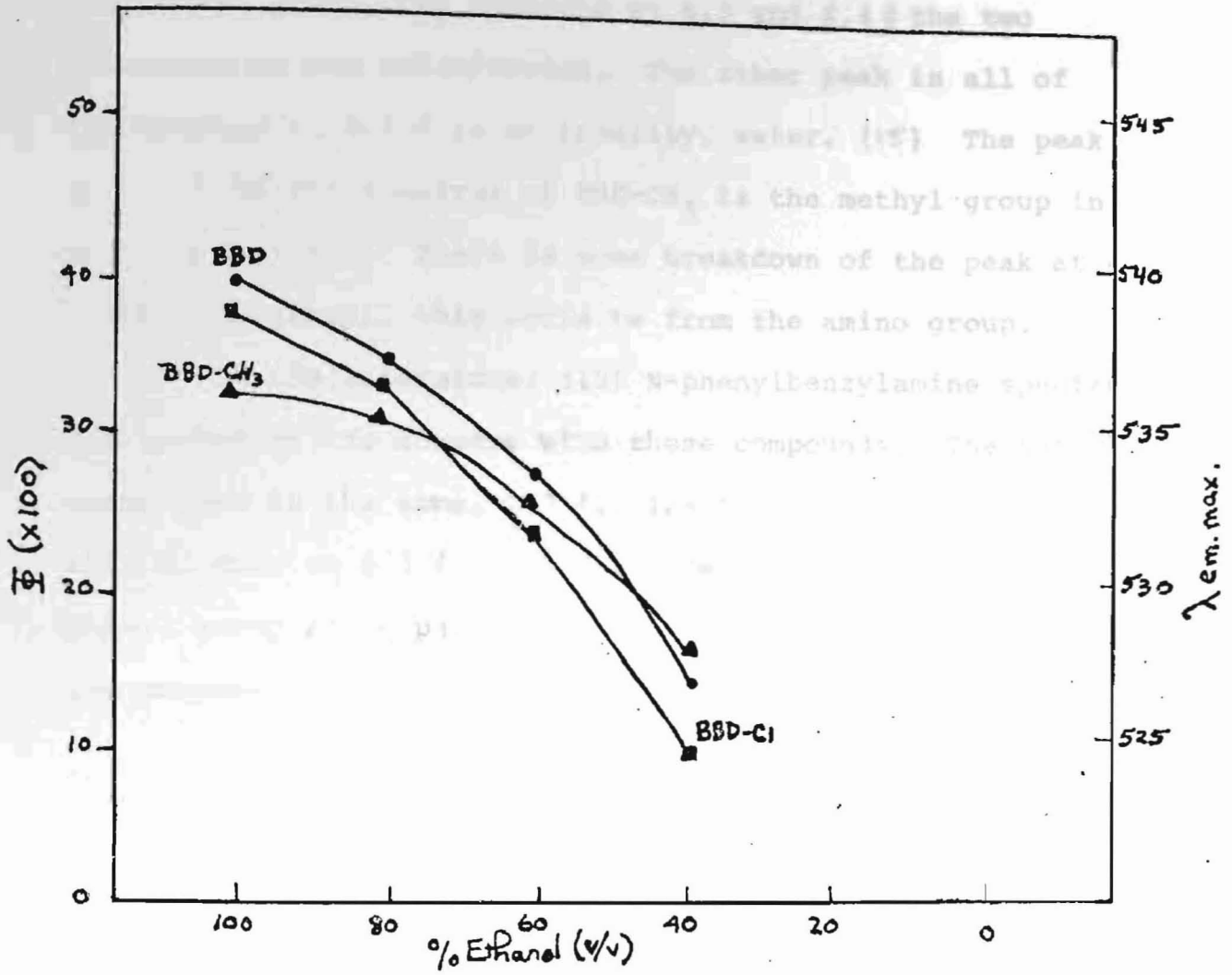


Figure 21. Quantum yield and emission maximum of BBD, BBD-CH_3, BBD-Cl in ethanol-water mixtures and dimethylformamide-water mixtures.

identified. The three compounds had similar spectra with the singlet at 4.78 the methylene group, the singlet at 7.3 δ the benzyl group, the doublets at 6.3 and 8.4 δ the two hydrogens on the benzofurazan. The other peak in all of the spectra at 3.2 δ is an impurity, water. (15) The peak at 2.2 δ on the spectrum of BBD-CH_3 is the methyl group in the para position. There is some breakdown of the peak at 4.7 (methylene group), this could be from the amino group.

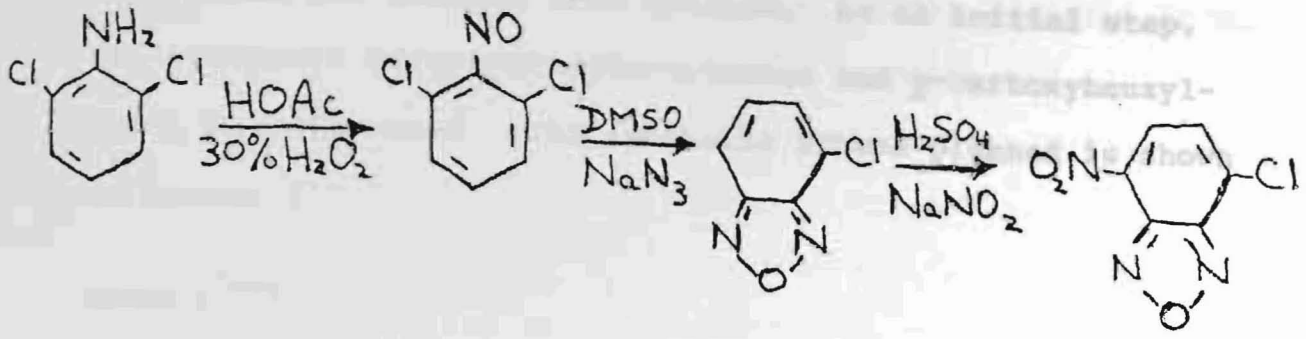
In the literature, (15) N-phenylbenzylamine spectrum was looked at, to compare with these compounds. The benzyl group peak is the same, 7.3 δ. The methylene peak is shifted down to 4.2 δ which could be the effect of the phenyl group as compared to the benzofurazan. These spectra are probably the first NMR spectra of these compounds.

Outline of Synthetic Work

Preparation of NBD-Cl (11)

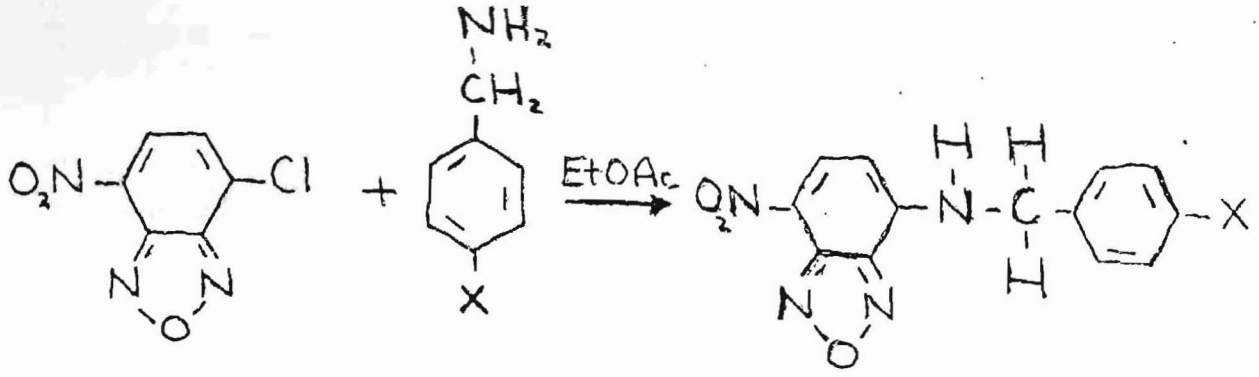
The preparation of 4-chloro-7-nitrobenzofurazan was the first part of the effort to prepare analogs of 7-(benzylamino)-4-nitrobenzofurazan. The synthesis of 4-chloro-7-nitrobenzofurazan was started with 2,6-dichloroaniline which upon treatment with 30% hydrogen peroxide gives 2,6-dichloro-nitrosobenzene (cf. Scheme I). This compound was then allowed to react with sodium azide to form 4-chlorobenzofurazan. This crude compound was converted to 4-chloro-7-nitrobenzofurazan upon treatment with sulfuric acid and sodium nitrite.

Scheme I



The preparation of the fluorescent probe molecule was accomplished by refluxing 4-chloro-7-nitrobenzofurazan with the appropriate benzylamine in ethyl acetate (cf. Scheme II).

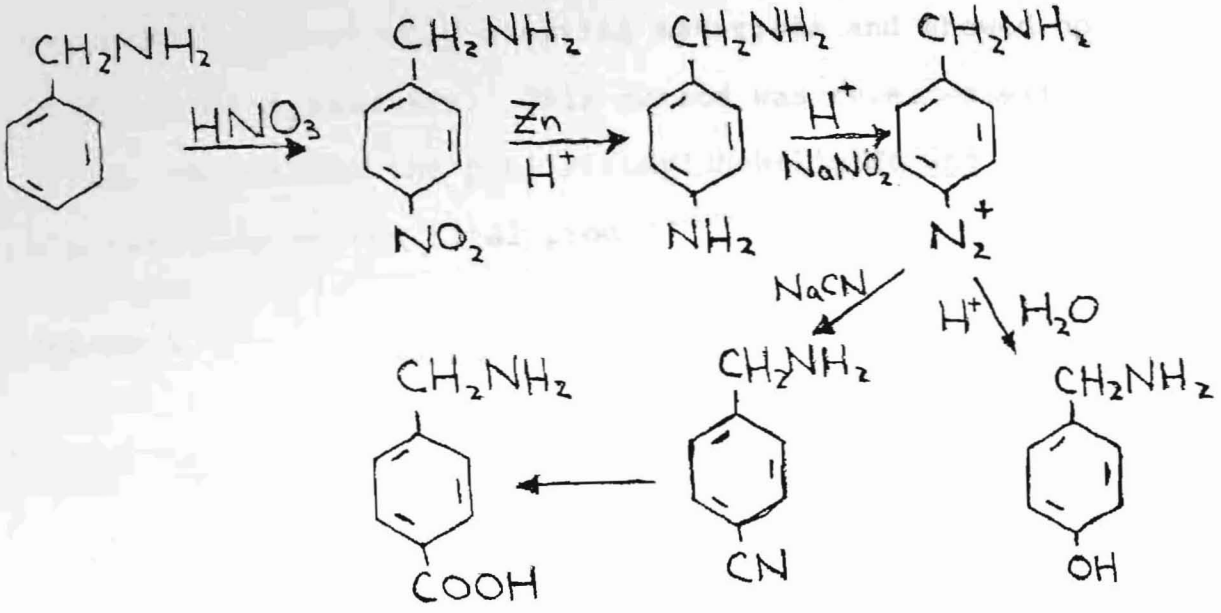
Scheme II



The derivatives of 7-benzylamino-4-nitrobenzyofurazan that were to be synthesized were those that are structural analogs of known inhibitors of selected enzymes (for example, benzoic acid is an inhibitor of D-amino acid oxidase). (1) Thus, the preparation of 7-(p-carboxylbenzyl-amino)-4-nitrobenzofurazan was attempted. This compound

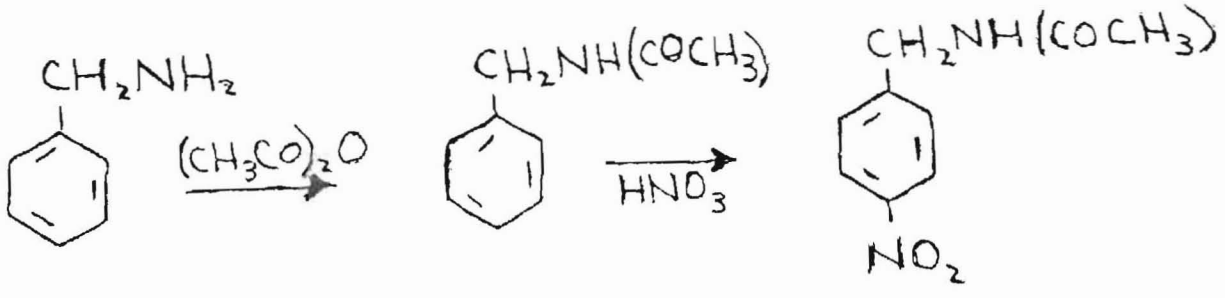
was selected as a model for an active-site directed fluorescent probe for D-amino acid oxidase. As an initial step, the synthesis of p-hydroxybenzylamino and p-carboxybenzylamine was attempted. The synthetic scheme planned is shown in Scheme III.

Scheme III



The synthesis of p-nitrobenzylamine from benzylamine proved to be difficult. A method by C. Paal and H. Sprenger (12) was attempted (cf. Scheme IV).

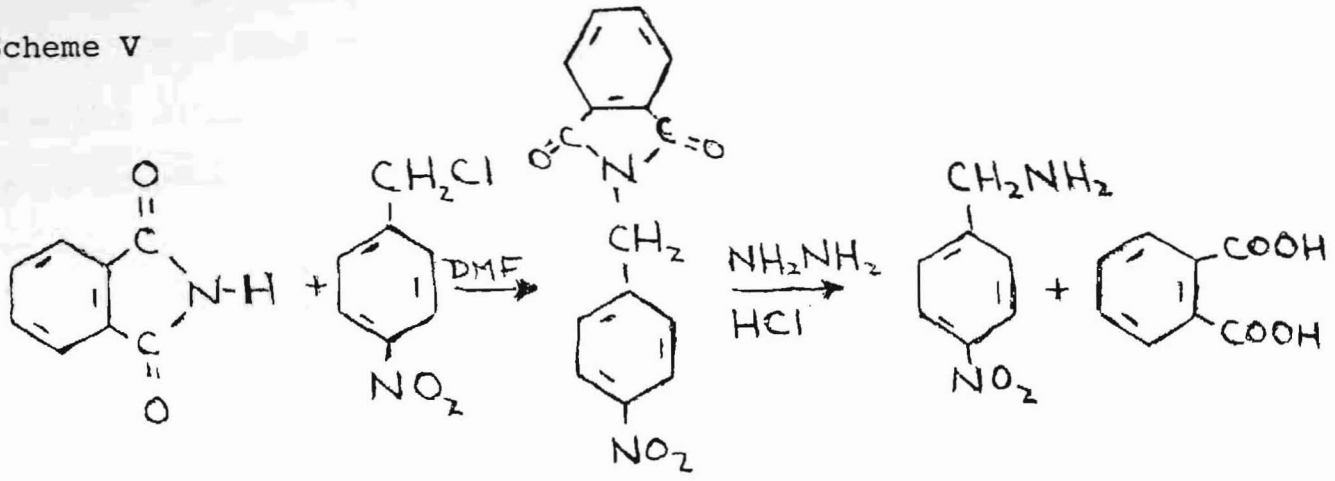
Scheme IV



The nitration step to form p-nitrobenzylacetamide was not selective enough so that not only was p-nitro compound formed but all the isomers. These could not be separated.

Another pathway to form the p-nitrobenzylamine was tried using a method described by L. I. Smith and O. H. Emerson (13) (cf. Scheme V). The phthalimide derivative of p-nitrobenzylchloride was attempted in dimethylformamide. The reaction gave only starting materials and showed no evidence of a reaction. This method was repeated without trying to isolate the p-nitrotolylphthalimide and taking the reaction to the final product. No product was obtained.

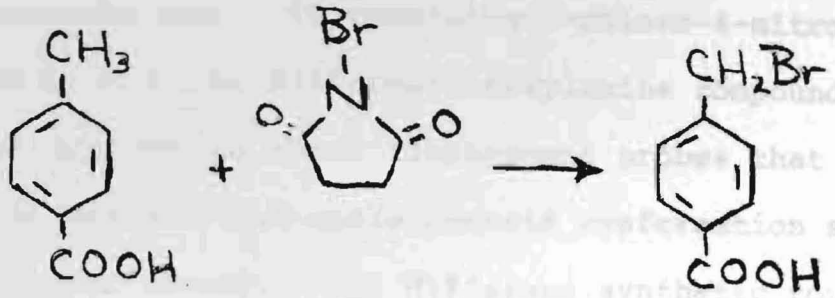
Scheme V



A third pathway that was tried was a method by F. L. Greenwood, M.D. Kellert, and J. Sedlak (14) to form the p-carboxybenzylamine from p-toluic acid (cf. Scheme VI). The product that was to be synthesized was α-bromo-p-toluic acid using N-bromosuccinimide and dibenzoyl peroside in carbon tetrachloride. There was no product formed in this reaction. The synthesis of this compound

may be accomplished if the methylester of p-toluic acid is used as the starting material. (16)

Scheme VI



Suggestions for Further Study

The most important compounds still to be synthesized are the fluorescent probes of p-hydroxybenzylamine and p-carboxybenzylamine. Other compounds could be looked at to see if they would be good fluorescent compounds similar to the derivatives of 7-benzylamino-4-nitrobenzyfurazan. An analog of these compounds with a sulfonic acid group in the paraposition would be important. Sulfonic acid groups could increase the solubility of the compound. Solubility is critical so that a readable fluorescence signal with normal light sources and detectors can be picked in studies of proteins. Also sulfonic acid groups can be modified or removed so that a new series of derivatives of 7-benzylamino-4-nitrofurazan could be construed. Also primary and secondary amines could be investigated.

CONCLUSION

The purpose of this study was to prepare and characterize some derivativ-s of 7-(benzylamino)-4-nitrobenzofurazan. By combining 7-chloro-4-nitrobenzofurazan with three different benzylamine compounds, we were able to synthesize three fluorescent probes that might be used for protein and nucleoprotein conformation studies.

Even though three different synthetic routes were tried, the p-carboxy derivative eluded us. On the positive side, two new compounds were synthesized. Since these compounds have such low solubility, no NMR spectra was given before in the literature. The NMR spectra of this study gave an additional identification of the compounds.

The quantum yield studies of these compounds indicated that the p-substituent has a slight effect on the spectral properties. This suggests that the p-substituent makes the compound more sensitive to solvent polarity which is useful in protein conformation studies. This groundwork will be helpful for the preparation of p-(carboxybenzylamino)-4-nitrobenzofurazan and the study of D-amino acid oxidase.

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